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CHARACTERIZATION OF INFECTIOUS PANCREATIC NECROSIS VIRUS ASSOCIATED WITH LAKE TROUT *(SALVELINUS NAMAYKUSH)* IN CORNWALL LAKE, ALBERTA

by

K. M. Shankar

©

A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY
EDMONTON, ALBERTA
FALL, 1991

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ABSTRACT

In 1984 an IPN virus was detected unexpectedly in feral lake trout from Cornwall Lake, Northern Alberta. A study was undertaken to determine the incidence of the virus in the lake trout population, its pathogenicity to lake trout and other salmonids, and its antigenic and molecular relationship to other IPN viruses in Canada.

The prevalence of the virus infection in lake trout of Cornwall Lake was found to be 44.4% with the virus found mainly in the pyloric caeca and intestine. Experimental infection by immersion of salmonid fry indicated that the virus is highly pathogenic to brook trout, less so to rainbow trout and non-pathogenic to its natural host, the lake trout. However, the virus persisted for up to 2-3 months in lake and rainbow trouts that survived the infection.

Cross neutralization studies using rabbit antisera indicated that the virus is similar to that found in Arctic char from the Northwest Territories, but not to IPN virus from Jasper. An analysis of the polypeptides of these isolates in 10% SDS PAGE revealed that the VP2 polypeptide of lake trout and Arctic char IPN virus consists of two unequal bands, which differ in their molecular weight.

A panel of fifteen monoclonal antibodies reacting with different epitopes on the VP2 polypeptide of lake trout IPN virus was produced to study the antigenic relationship of the virus. Epitope analysis of Canadian isolates by immunodot and neutralization assays showed three monoclonal antibodies specific to lake trout IPN virus. By using these specific monoclonal antibodies, isolates from a series of recent IPN outbreaks in Alberta were identified as lake trout IPN virus. Overall, the panel of 15 antibodies clearly differentiated lake trout virus from that of the proposed Canadian IPN virus serotypes.

In order to study the molecular relationship of lake trout IPN virus to other isolates, DNA of the virus was synthesized by the polymerase chain reaction using primers derived from Jasper IPN virus. The DNA was sequenced either directly or after cloning, by the dideoxy chain termination method. Comparison of the gene encoding the VP2 polypeptide of lake trout and Jasper IPN viruses from nt 474 to 676 and 1200 to 1300 showed a homology of 80-84% in nucleotide sequence and 100% in deduced amino acid sequence.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
cDNA	Complementary DNA
CHSE-214	Chinook salmon embryo cell line
CPE	Cytopathic effect
ddd H_2O	double distilled deionised water
DEPC	Diethylpyrocarbonate
DI particle	Defective interfering particle
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscope
FAT	Fluorescent antibody test
FBS	Fetal bovine serum
HAT	Hypoxanthine, aminopterin and thymidine
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	Horseradish peroxidase
HT	Hypoxanthine and thymidine
IFAT	Indirect fluorescent antibody test
Ig	Immunoglobulin
IgG	Immunoglobulin G
IHNV	Infectious hematopoietic necrosis virus
IPN	Infectious pancreatic necrosis
IPNV	Infectious pancreatic necrosis virus
K	Kilodalton
MAb	Monoclonal antibody
MEM	Minimal essential medium
MOI	Multiplicity of infection
mRNA	Messenger RNA
mw	Molecular weight (s)
nt	Nucleotide (s)
ND_{50}	50% cell culture protection dose of antiserum
OPD	0-phenylenediamine dihydrochloride
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFU	Plaque forming unit
RNase	Ribonuclease

RPMI	RPMI 1640 medium
RTG-2	Rainbow trout gonad cell line
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
$TCID_{50}$	50% tissue culture infectious dose
TNE	Tris-EDTA NaCl buffer
UV	Ultraviolet

CHAPTER 1

General Introduction

1.1 Infectious pancreatic necrosis of fish

Infectious pancreatic necrosis (IPN) is a highly contagious disease, primarily of young salmonids cultivated under intensive rearing conditions. The disease was first reported in 1940 in Canada by M'Gonigle (1941) in brook trout (*Salvelinus fontinalis)* fry as an acute catarrhal enteritis. Subsequently, outbreaks of clinically identical disease in brook trout hatcheries in the U.S.A. were investigated by Wood *et al.* (1955). Their transmission experiments and histopathological examination led them to name the disease infectious pancreatic necrosis and to suggest a viral etiology. The viral nature of IPN was confirmed by Wolf *et al.* (1960), who isolated the virus in fish cell cultures and reproduced the disease in brook trout fry. Today IPN disease is widespread in North America, Europe, and Asia (MacKelvie and Artsob, 1969; Sano, 1971 and Ljungberg and Jorgenson, 1972). IPN virus (IPNV) has also been isolated from rainbow trout *(Oncorhynchus mykiss)* in Chile (McAllister, 1984) and from goby (*Oxyleotris marmoratus*) in Taiwan (Chen *et al.*, 1985). Thus, IPNV has a greater geographical distribution than any of the other viruses causing disease in fish.

1.2 Physical characteristics of IPNV

IPNV belongs to the family *Birnaviridae* which includes viruses possessing bisegmented ds RNA. The virus has icosahedral symmetry and has a diameter of 60-70 nm (Lightner and Post, 1969; Chang *et al.*, 1978). The single capsid of the virus is composed of 180 structural subunits shared by 92 pentagonal and hexagonal capsomers (Underwood *et al.*, 1977). The virion has a sedimentation coefficient of

430-440 S, with a mw of about 55×10^6, and a buoyant density of 1.32-1.33 g/ml in CsCl (Cohen *et al.*, 1973; Dobos, 1976; Dobos *et al.*, 1979).

1.3 Molecular biology of IPNV

The virus protein consists of four polypeptides VP1, VP2, VP3, and VP4 with approximate mw of 105,000, 55,000, 30,000, and 28,000 daltons respectively, in the proportion of 3:24:17:15 (Chang *et al.*, 1978; Macdonald and Dobos, 1981; Mertens and Dobos, 1982).

The genome, which represents 8.7% of the total virion mass is comprised of two segments of double-stranded RNA of mw 2.5×10^6 each with a GC content of 54%. The two segments of the genome correspond to two RNA molecules each with different nucleotide sequences, and hence are unrelated (Macdonald *et al.*, 1977). The two RNA genome molecules each have a sedimentation velocity of 14 S in sucrose gradients, a buoyant density of 1.60 g/ml in $CsSO_4$, a pyrimidine and purine ratio near unity, and an average length of 0.92 μm (Macdonald and Yamamoto, 1977).

In order to determine which proteins are encoded by each segment of the genome, Macdonald and Dobos (1981) produced hybrids possessing mutually exchanged RNA segments by crossing ts mutants belonging to two serotypes. An analysis of the proteins of each hybrid showed that the larger RNA (segment A) coded for the major capsid protein (VP2), the internal protein (VP3), and a non-structural protein (NS), whereas the smaller RNA (segment B) coded for the 100,000 dalton protein (VP1), which is a putative RNA dependent RNA polymerase.

Huang *et al.* (1986) constructed a physical map for the genome of the Sp serotype of IPNV. The single-stranded RNA transcribed by the subclones were translated in a rabbit reticulocyte lysate translation system. The four proteins of IPNV were identified by immunoprecipitation and SDS-PAGE. Based on the study they derived the linear gene order for the viral proteins encoded by segment A as pVP2, NS and VP3.

Further work on the proteins encoded by segment A was carried out by Nagy *et al.* (1987), who mapped the large RNA (segment A) of IPNV based on studies by hybrid arrested translation. The large ds-RNA segment which encodes three of the four virus coded polypeptides (pre-VP2, VP3 and NS) was cloned. The plus and minus RNA strands were separated and identified. Hybrid arrested condition which blocked the 5' two third of A+ RNA allowed in vitro synthesis of only VP3. Hybridization of RNA to DNA representing the 5' half of A+ RNA allowed synthesis of both NS and VP3 but not of VP2. *In vitro* translation of A+ RNA yielded all three polypeptides. Therefore, they concluded that the order of the three polypeptides on the A+ RNA is 5'-VP2-NS-VP3-3'.

More information on proteins encoded by segment A was available from the study of Duncan *et al.* (1987). They subcloned full length and truncated segment A specific cDNA into plasmid vectors, and produced run-off translation products *in vitro*. The translated products were analysed by SDS-PAGE. The cDNA directed the synthesis of authentic VP2, VP3, and NS polypeptides, as well as a number of previously undescribed polypeptides. A 101,000 dalton polypeptide was isolated and shown to be the unprocessed IPNV polyprotein. The NS polypeptide appeared to be a virus encoded protease responsible for cleavage of VP2 from the polyprotein.

Duncan and Dobos (1986) sequenced segment A of Ja IPNV and showed that it has one large open reading frame (ORF) encoding a precursor polyprotein. Manning *et al.* (1990) showed with a series of deletion transcripts from segment A of Sp IPNV that the NS gene is required for proteolytic processing of polyprotein.

Further, Manning and Leong (1990) demonstrated that the precursor polyprotein is also processed when the entire IPNV A segment is expressed in *Escherichia coli*. Their results suggested that the NS protease is autocatalytic and solely responsible for both the VP2/NS and NS/VP3 cleavages.

In order to develop a subunit vaccine useful for prevention of IPN disease the gene encoding the outer capsid protein (VP2) was expressed in *Escherichia coli*, by Lawrence *et al.* (1989). A 926 bp viral cDNA encoding an N-terminal truncated VP2 was cloned into the PWR 590 expression plasmid resulting in a C-terminal extension of truncated *E. coli* beta- galactosidase under the control of lac promoter. A 100 K beta-Gal-VP2 fusion protein was expressed within 4 h after induction. The fusion protein reacted in Western blot with both rabbit anti beta-Gal and with neutralizing mouse anti-VP2 monoclonal antibody. Sera of rabbits immunized with semipurified fusion protein reacted with the VP2 polypeptide in Western blot and with the intact purified virus in ELISA, and also neutralized IPNV infectivity in the plaque reduction assay.

Havarstein *et al.* (1990) sequenced the large ds-RNA segment of N1 Norwegian strain of IPNV, and compared it to that of Ja-IPNV and the 002-73 strain of infectious bursal disease virus of chickens. The cDNA and the deduced amino acid sequence indicated that the precursor protein of the major polypeptide VP2 is highly conserved at the N and C termini, whereas an internal segment I from nt 550-1014 (aa 183-338) shows greater diversity between strains. Their study indicated that the internal segment carries the serotype specific epitope of birnaviruses.

IPNV possess a genome-linked protein (VP_G), as demonstrated by Persson and Macdonald (1982). Double-stranded RNA, extracted by a method without proteinase, exhibited a lower electrophoretic mobility in SDS-polyacrylamide and agarose gels, lower buoyant density, and a marked tendency towards aggregation. Therefore, they concluded that a small amount of protein is tightly bound to the RNA. Further, they separated and purified a protein having a mw of 110,000 daltons by subjecting IPNV-RNA to RNAse III and RNAse A. The protein, as revealed in electron micrographs, was found to bind to the ends of RNA segments at an average of 1.4 molecule per segment.

Mertens *et al.* (1982) demonstrated the presence of RNA dependent RNA-polymerase in purified IPNV. It was found that 50% of the polymerase products remained associated with the ds RNA template and the rest as extravirion ss RNA broken down to 5 S and 7 S fragments by virus-associated RNases.

1.4 Propagation of IPNV

Growth of IPNV in cell culture can be achieved in a variety of continuous cell lines from teleost fishes at temperatures below 24°C (Lannan *et al.*, 1984). Among CHSE-214, FHM and RTG-2 cell lines, the former has proven to be very effective in detection and propagation of IPNV (Kelly *et al.*, 1978). Cell lines of amphibian, avian or mammalian origin do not support IPNV replication.

A single cycle of replication takes 16 to 20 h at 22°C, resulting in a characteristic cytopathic effect (CPE) (Malsberger and Cerini, 1965). Virus could be detected 4 h after infection of cell cultures. Roberts and Dobos (1983) observed maximum yields of infectious particles at 20°C. The virus failed to replicate at 28°C and neither virus specific mRNA nor polypeptides could be detected when infected cells were maintained at 28°C. Analysis of infectious virus production, virus-specific RNA and polypeptide synthesis following shift from permissive (20°C) to non-permissive (28°C) temperature at varying times after infection indicated that multiple temperature sensitive steps are involved in the inhibition of virus replication.

1.5 Characteristics of IPN disease

The clinical disease has usually been observed under circumstances of hatchery cultivation of fish, because it is difficult to monitor disease in the wild. In hatchery populations, the first sign of outbreak is a progressive increase in daily mortalities in fry, particularly in the rapidly growing individuals. Clinical signs of IPN disease include darkened body pigmentation, a distended abdomen, and most typically, a corkscrewing and spiralling swimming motion in severly affected fish. Exophthalmia may be present. Internally, there is frequently a large amount of whitish mucus and sloughed epithelial cells within the lumen of the stomach and intestine. There may also be signs of petechial haemorrhage over the anterior visceral mass and the liver and spleen are often enlarged and pale.

The cumulative mortality due to IPN disease varies considerably in different outbreaks. In the worst situation an epizootic may persist for many weeks and cumulative mortality may reach 90% or more (Wolf *et al.*, 1960). Virus levels in visceral organs often reach a peak of 10^7-10^9 infectious particles/g of tissue and large quantities of virus are excreted by moribund fry through intestinal discharges.

An important feature of IPN disease is the marked increase in resistance of the host with age, the most susceptible fish being early feeding fry and high resistance being reached by the age of 5 to 6 months. However, rare cases of disease have been reported in yearling fish (Elhazary *et al.*, 1976; Roberts and McKnight, 1976). It has been generally observed that fish surviving acute infection at the fry stage will have reduced growth rates (McKnight and Roberts, 1976; Munro and Duncan, 1977).

A comprehensive report of the sequential histopathology of the naturally occuring IPN disease has been given by McKnight and Roberts (1976). Their study revealed that the pancreas is the major target organ with severe necrosis of the pancreatic acinar cells, together with nuclear pycnosis and occasional intracytoplasmic inclusions. Islet tissue may or may not be affected. The mucosa of the pylorus, pyloric caeca, and anterior intestine show an acute enteritis in the form of necrosis and sloughing of epithelial cells. Electron microscopy of the infected tissues has revealed large crystalline arrays of the virus within the cytoplasm of pancreatic cells showing advanced pathological changes approaching necrosis (Lightner and Post, 1969; Ball *et al.*, 1971).

Swanson and Gillespie (1982) experimentally infected yearling rainbow and brook trouts with IPNV by intraperitoneal injection and by bath immersion, respectively. The virus was recovered consistently from plasma and mononuclear cells enriched blood fractions of rainbow trout from 1 to 19 d post-infection (DPI). In the case of brook trout the virus was detected in the plasma from 3 to 40 DPI and from 7 to 40 DPI in the mononuclear fraction. The variation in viraemia between the two treatments may be due to two different routes of infection. Extensive viral replication takes place in pancreatic tissue although viral antigen and slight pathological changes were found in kidney, intestine, and livers of some fish.

In experimental infections, Okamoto *et al.* (1984) studied the correlation in the quantity of IPNV in rainbow trout fry and the severity of the disease process. Following exposure to the virus, signs of disease and first mortality appeared 5 to 6 DPI. During the incubation period of 4 d, the virus quantity in tissues was low ($< = 10^{4.1}$ $TCID_{50}/g$). Clinical disease was observed at $10^{5.1}$ $TCID_{50}/g$ and mortality of the infected fry began at $> = 10^{5.1}$ $TCID_{50}/g$. The earliest they could recover the virus from infected fry was 10 h post infection and at this time only a small number of fry were infected (10-15%). However, between the 2nd and 4th DPI, viral titers of 10^3 to $10^{4.0}$ $TCID_{50}$/g were detected in most of the infected fry.

1.6 Immune response in fish infected with IPNV

Jorgensen (1973) found that rainbow trout artificially immunized with IPNV produced neutralizing antibody titers of 1:2800 6 weeks after inoculation. A maximum titer of 1:24,000 was found 12 weeks after inoculation. The antibodies produced were sensitive to incomplete reduction and alkylation, and based on this he concluded that IPNV neutralizing activity in trout is associated with an IgM-like immunoglobulin. Kelly and Nielsen (1990) immunized adult rainbow trout with inactivated, concentrated IPNV of different serotypes to study the neutralization specificity of antibodies. Sera containing high titers of antibody were obtained and cross reaction in neutralization tests was found to be generally low between serotypes.

The relationship between virus and neutralizing antibody levels in IPNV carrier fish is not clear. Yamamoto (1975) noticed a decline in carrier status accompanied by elevated levels of neutralizing antibody in sera of naturally infected rainbow trout during a 5 month observation. In contrast, Reno *et al.* (1978) did not find an inverse relationship between neutralizing antibodies and virus titers in experimentally induced carriers. They induced the IPNV carrier state in yearling and juvenile trout after infection by immersion or intraperitoneal injection. Infection was sustained for a longer period at low virus level along with low levels of neutralizing antibodies.

In addition to salmonids, non-salmonids may also produce neutralizing antibody against IPNV. Wechsler (1987) recorded neutralizing antibody against IPNV in striped bass (*Morone saxatilis*) following intraperitoneal injection with the virus.

1.7 Persistence of IPNV in fish

Salmonids that survive IPNV infection become non-clinical carriers, probably for life (Yamamoto and Kilistoff, 1979), and excrete various quantities of virus in their feces and seminal or ovarian fluids (Wolf *et al.*, 1968; Billi and Wolf, 1969; Frantsi and Savan, 1971a). The virus is also detectable in the viscera of carriers, particularly in the kidney. The incidence and persistence of the carrier state has been found to be higher in brook trout than in rainbow trout (Wolf *et al.*, 1968; Yamamoto, 1974).

It has been speculated that defective interfering particles (DI) may play a major role in determining the persistence of the carrier state for IPN. IPNV can produce DI particles in cells *in vitro*, which interferes with production of homologous standard infectious virus and leads to persistent infection in cell cultures (Nicholson and Dunn, 1974; Macdonald, 1978; Hedrick *et al.*, 1978).

Nicholson and Dexter (1975) described possible interference in the isolation of IPNV from carrier fish due to the presence of DI particles in host tissues. Hedrick *et al.* (1978) suggested that the ability of IPNV to persist in carrier trout may be the result of a balance between DI particles and standard virus particle production in the host. Later, Hedrick *et al.* (1982) studied this persistence in carrier brook trout and in persistently-infected RTG cell lines. Viral persistence *in vitro* and *in vivo* was characterized by a fluctuating release of infectious virus ($< 10^{1.6}$ to $10^{6.0}$ $TCID_{50}$/g or ml) in the absence of external signs or cytopathic effect (CPE). Infectious center assays indicated that virus was produced by less than 1% of the cells. The role of antibody *in vitro* and *in vivo* is not clear. The similarity of viral persistence in cell lines and carrier brook trout suggested that the same virus-host cell interactions occur *in vitro* and *in vivo*.

Yu *et al.* (1982) isolated the virus from leucocytes of carrier brook trout, by co-cultivation methods. However, they found that when normal fish leucocytes were subjected to IPNV, less than 0.01% of them were infected with the virus, and hence concluded that leucocytes are not the major site of replication.

Knott and Munro (1986) observed a suppressed response to phytohemagglutinin stimulation from leucocytes of Atlantic salmon carriers compared with uninfected controls. IPNV was isolated from 41% of stimulated leucocytes culture supernatants, while only 6% of the unstimulated cultures were found to be positive. Phytohemagglutinin (PHA) stimulates the DNA synthesis machinery, which indirectly activates virus multiplication, and hence is a good method of diagnosis. They observed a significant degree of inhibition using ^{3}H-thymidine incorporation studies. The host cells are thus suppressed in terms of DNA synthesis, leading to inhibition of viral replication and the establishment of carrier status.

Smail and Munro (1985) had observed that persistant infection in Atlantic salmon adults did not significantly affect their growth. They observed large scale necrosis of the pancreas but speculated that it would not have much impact on the production of digestive enzymes in adult animals.

1.8 Transmission of IPNV

Snieszko *et al.* (1959) first suggested that there is evidence for vertical transmission of IPN. Further work has shown that eggs from female carriers are infected or intimately contaminated with the virus and vertical transmission to offspring readily takes place (Wolf *et al.*, 1963; Bullock *et al.*, 1976). Fijan and Giorgetti (1978) isolated IPNV from eyed eggs of rainbow trout and they considered that the virus was most likely harboured inside the egg. Ahne and Negele (1985) studied the surface of fertilized eggs of rainbow trout and Arctic char, before and after water hardening, by scanning electron microscopy. After hardening, the smooth surface of the egg shell changes to coarse, with lobes and pores providing a better anchorage for the virus. The virus thus adsorbed is protected from disinfection with iodine. Apart from virus transmission on the surface, sperm may also carry the virus (Dorson and Torchy, 1985). A strong adsorption of IPNV to trout spermatozoa was also documented by Mulcahy and Pascho, (1984).

Bootland *et al.* (1989) induced and maintained the IPNV carrier state in yearling brook trout for 76 weeks by intraperitoneal injection with the virus. Later they demonstrated vertical transmission of IPNV in 2 % of the progeny.

Horizontal transmission of IPNV can take place either by contact with IPNV contaminated water (Mulchay *et al.*, 1983) or by cannibalism as observed with fry of striped bass (*Morone saxatilis*) (Wechsler *et al.*, 1987)

The possibility that warm blooded animals may act as mechanical vectors for IPNV has been demonstrated by Peter and Neukirch (1986). Following oral inoculation with IPNV infected fish, infectious virus was detected in herons *(Ardea cinerea*) up to 7-12 d depending on the amount of infected food consumed.

1.9 Virulence of IPNV

The severity and cumulative mortality of IPNV infection in trout hatcheries can vary considerably, from over 90% to insignificant levels. The virulence of infection in salmonids depends on a number of factors relating to the host, the virus, and the environment.

The virulence of IPNV depends to a great extent on the particular host species it infects. Within the salmonid species there is a marked difference between species in the degree of susceptibility to IPN. Brook trout, rainbow trout, and amago trout (in Japan) are the species most severely affected by IPN, whereas Atlantic salmon and brown trout are affected to a lesser extent. There are no reports of natural clinical IPN in other salmonids despite the detection of a natural viral infection. Comparative susceptibility of various salmonid species has been studied by several workers. Parisot *et al*, (1963) reported that chinook salmon (*Oncorhynchus tschawytscha)*, kokanee salmon (*O. nerka*) and coho salmon (*O. kisutch*) are resistent to disease when inoculated with an isolate from brook trout. Sano (1973) reported that IPNV from rainbow trout was almost as virulent for two *Onchorhynchus* species, amago (*O. rhodurus*) and himemasu (*O. nerka*), as it was for rainbow trout fry. Fry of Atlantic salmon fail to get disease, when subject to the rainbow trout isolate despite evidence of virus replication (Swanson and Gillespie, 1979). Variability in susceptibility to IPN disease among strains of a fish species, for example rainbow trout, also exists (Elazhary *et al*., 1976).

The important influence of age of the host on the virulence of virus has been recognized since the first field observation of IPN disease (M'Gonigle, 1941). Susceptibility to IPN disease decreases steadily with age and resistance to disease is reached by the age of 5-6 months. Fish older than this can be infected by the virus, but show no clinical signs of disease, even following intraperitoneal infection. Such fish develop a transient non-clinical infection, which results in high levels of neutralizing antibody and ultimately loss of virus from fish tissue (Wolf and Quimby, 1969). Frantsi and Savan (1971b), in an experimental infection of brook trout within a 60 d observation period, observed a cumulative mortality of 85% in 1 month old, 73% in 2 month old, 45% in 4 month old and negligible mortality in 6 month old fry. Subsequently, Dorson and Torchy (1981) observed that in rainbow trout, cumulative mortality within 2 months of infection decreased steadily from 70% in fish infected when 1 or 2 weeks old to a negligible percentage in fish infected at 20 weeks old. A graph of cumulative mortality against age at the time of infection shows an almost linear relationship. They concluded that, using mortality as a criterion, rainbow trout cease to be susceptible to IPN when 15-20 weeks old, even though they may be actively infected by the virus following exposure.

Environmental factors have a profound influence on outbreak of fish disease. Under stresses such as poor water quality, overcrowding and handling, disease outbreaks are common apparently due to suppression of the fish immune system. The depression of the immune response to IPNV in blue gouramy (*Trichopterous sp.*), when held under overcrowded conditions, has been demonstrated by Perlmutter *et al.* (1973). Stresses such as low dissolved oxygen in water and transportation cause a marked enchancement of the replication of IPNV in carrier fish (Frantsi and Savan, 1971a; Roberts and McKnight, 1976).

Among the various environmental parameters, water temperature has a profound influence on the development of disease in poikilotherms. Both fish and pathogen have their optimal temperature range and the outcome of disease can be indicated by shifts in water temperature. Following experimental infection of brook trout fry with IPNV, Frantsi and Savan (1971) obtained cumulative mortalities of 74% at 10°C, 46% at 15.5°C and no mortality at 4.5°C. Dorson and Torchy (1981) observed that cumulative mortality in rainbow trout fry infected with the virus, was much less at 6°C than at 10°C and was almost totally suppressed at 16°C. In most trout hatcheries, where water is taken from a spring or bore hole, the supply temperature normally stays from 8 to 12°C. Lapierre *et al.* (1986) also observed a decreased mortality of brook trout fry due to IPN infection at 5°C compared to that at 10°C and 15°C.

Virulence of IPNV strains vary. Among the 3 serotypes of IPNV, Ab (Denmark), Sp (Denmark) and VR 299 (North America), the latter two are more virulent than IPNV-Ab. Hill and Dixon (1977) compared the virulence of IPNV-Sp strains isolated from diseased fry and non-clinical carriers. It was found that most of the virus isolates had approximately equal virulence, irrespective of whether they were isolated from diseased fry or from carriers.

Dorson *et al.* (1978) demonstrated that after a relatively low number of passages through fish cell culture, virulent IPNV developed sensitivity to neutralization by normal trout serum, and at the same time became avirulent for rainbow trout fry. The neutralization of cell culture-adapted virus by normal rainbow trout serum has been shown to be due to the presence of an antibody-like non-virus induced protein having a sedimentation coeffient of 6 S (Kelly *et al.*, 1985).

However, Dixon (1987) demonstrated that inhibition of IPNV infectivity can also be due to a factor other than the 6 S factor. He used IPNV that was resistant to neutralization by the 6 S factor to study in detail inhibition of IPNV infectivity by tissue extracts of healthy rainbow trout. Extracts of liver, kidney, spleen, and whole fry inhibited plaque production of IPNV in cell culture. The mode of inhibition was not known, although it appeared not to be manifested at the cellular level, as pretreatment of cell cultures with tissue extract did not inhibit plaque production. The inhibition may be due to reduction of virus attachment to the cell surface or to aggregation of virus and thus reduced infectivity units.

1.10 Diagnosis of IPNV infection

Detection of IPNV by cell culture is the most commonly used and reliable method compared to other detection methods developed so far. Methods such as co-cultivation of leucocytes and kidney cells from infected fish with CHSE-214 cells is also known to increase sensitivity of detection. In a persistently infected population of trout 75% were found to be IPNV positive by leucocyte co-cultivation method whereas by ordinary cell culture methods 98% were positive (Yu *et al.* 1982). This study indicated blood samples can be used for detection of IPNV in carrier populations.

Tu *et al.* (1974) standardized the immunofluorescent assay of IPNV in infected cell culture. They found a linear relationship between the relative concentration of virus in the inoculum and number of fluorescent cells in the first cycle of infection. Swanson and Gillespie (1981) evaluated the immunofluorescent antibody test for rapid detection of IPNV in acutely infected (10^5--$10^{7.5}$ $TCID_{50}$ /ml) rainbow trout fry. It is a specific test useful in rapid detection of virus at the acute infection stage, but it may not detect the virus efficiently at the early stages of infection.

Nicholson and Henchal (1978) evaluated the direct and indirect immunoperoxidase techniques for the identification of IPNV infected cells. Both methods were shown to be relatively simple, rapid, and efficient for specific identification.

Finlay and Hill (1975) used the complement fixation test for rapid typing of IPNV. The test could identify the isolate within 24 h of detection of CPE in cell culture, compared to 6-7 d with the serum neutralization test. Among the several sources of RBC tested, Cleator and Burney (1980) found that IPNV hemagglutinates RBC from mice but not from other mammals and birds. Hemagglutination was pH-dependent (optimum range 5.75-6.0) and positive results were obtained at 10^7-10^8 $TCID_{50}$ /0.1 ml.

Nicholson and Caswell (1982) used a solid phase, double-antibody enzyme linked immunosorbent assay (ELISA) for detection of IPNV in infected fish. They showed that ELISA could detect a minimum of $10^{5.5}$ $TCID_{50}$/ml of virus. They suggested that the method is rapid and inexpensive for detection of acute infection, but not advisable for virus detection in carriers. Later, Dixon and Hill (1983) used ELISA to demonstrate the presence of IPNV in infected fish. Virus antigen was detected in infected fry during and immediately following an epizootic. Though IPNV carriers could be detected by ELISA, the sensitivity of detection was not as high as that of isolation in cell culture. Out of 70 samples of rainbow and brook trout fry, 36 were found to be positive by cell culture, whereas only 10 were positive with ELISA. However, the test is a very rapid means of detection and could be used to serotype IPNV isolates, as major serotypes of IPNV (VR 299, SP and Ab) cross reacted at only a low level. Rodak *et al.* (1988) using a double antibody ELISA increased the sensitivity of detection by ELISA to $10^{2.0}$ $TCID_{50}$/0.1 ml of culture supernatant. They used IPNV antisera raised in rabbit and swine.

Kimura *et al.* (1983) used the coagglutination test using antibody-sensitized staphylococci as a rapid and simple serological test for diagnosis of IPN infection. A positive reaction (coagglutination) occured only with the antigen, the antiserum of which was used to sensitize staphylococci, and no cross reactions were observed. The agglutination test could also detect antigen in tissue extracts with relatively high sensitivity. The test can give positive results within 30 min and the amount of viral antigen required for a positive reaction is $10^{5.9}$ to $10^{7.7}$ $TCID_{50}$/ml. This is a rapid simple test requiring no special apparatus and hence ideal for field trials.

Dea and Elazhary (1983) employed counter immunoelectrophoresis for identification of IPNV, after isolating the virus in cell culture. The limit of detection of the method was 10^5 $TCID_{50}$/ml, and hence was not ideal for detection of virus in carrier fish.

Caswell-Reno *et al.* 1989 developed an enzyme immunodot assay for a rapid and specific identification of aquatic birnaviruses. The assay could detect 10^7

$TCID_{50}$ /ml of virus from cell culture supernatant. Hsu *et al.* (1989) used the immunodot method for detection of IPNV in infected cells and fish. The assay could be completed in 4 h and could detect virus within 3 h post infection of cells, provided the cells were infected at sufficiently high multiplicity of infection. The sensitivity of the assay was 10^4 to 10^5 $TCID_{50}$/0.1 ml or 2.5 ng of purified IPNV protein per dot.

Rimstad *et al.* (1990) detected IPNV in salmonid fish by the polymerase chain reaction (PCR). The sensitivity of the double nested PCR with magnetic separation of the amplified DNA was approximately 0.8 picograms of ds RNA.

1.11 Host range of IPNV

IPN disease outbreaks have been frequently recorded in brook and rainbow trout hatcheries. The disease has also been observed in hatchery reared cutthrout trout (*Salmo clarki*) (Parisot *et al.*, 1963) and amago (*Oncorhynchus rhodurus*) (Sano and Yamazaki, 1973) and himemasu (*Oncorhynchus nerka*) (Sano, 1973).

Natural infections with the virus, mostly as carriers, have been observed in a number of other salmonid species, namely Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta)* (Mackelvie and Artsob, 1969), Coho salmon (*Oncorhynchus kisutch*) (Wolf and Pettijohn, 1970), Arctic char (*Salvelinus alpinus*) (Souter *et al.*, 1984), Danube salmon (*Hucho hucho*) (Ahne, 1980) and lake trout (*Salvelinus namaykush*) (Larson, 1985; Terry MacFadden and Yamamoto, personal communication 1986). However, with the exception of Atlantic salmon and brown trout clinical disease does not appear in these hosts.

IPNV can also infect a wide variety of non-salmonids. These reports are based on neutralization of the isolates with reference antisera and in some with additional biophysical and biochemical characteristics. The first report of such an infection was in suckers (*Catastomus commersoni*) near a hatchery in Canada with a history of IPN (Sonstegard *et al.*, 1972). Later, during 1973-74, IPNV was isolated from several non-salmonids in the United Kingdom, namely carp (*Cyprinus carpio*), roach (*Rutilus rutilus*), bream (*Abramis brama*), and perch (*Perca fluviatilis*) (Hill, 1976). In experimental infections, all, except the isolate from perch, proved pathogenic to some degree for rainbow trout, producing the characteristic clinical signs of IPN. Munro *et al.* (1976) reported detection of IPNV in minnows (*Phoxinus phoxinus*) and lamprey (*Lampetra fluvialitis*) in a resident population in Scotland. Ahne (1978) reported isolation of the virus from moribund pike (*Esox lucius*). Adair and Ferguson (1981) have reported IPNV infection in gold fish (*Carassius auratus*), and discus fish (*Symphysodon discus*) in Northern-Ireland. The virus has been isolated from sand goby (*Oxyleotris marmorutus*) in Taiwan (Chen *et al.*,1985) and from common dab (*Limanda limanda*), a sole (Diamont *et al.*,1986).

Few cases of IPNV causing disease with specific clinical signs have been reported in non-salmonids. Sano *et al.* (1981) isolated IPNV associated with a major annual epizootic in farmed European eel (*Anguilla anguilla*) and Japanese eel (*A.japonica*). The virus isolate was avirulent in rainbow trout. However, the IPNV (Sp strain) isolated from healthy European elvers in France could cause typical IPN disease in rainbow trout but not in eel (Castric and Chastel, 1980). Stephen *et al.* (1980) isolated IPNV from large annual epizootics of spinning disease in natural stocks of Atlantic menhaden (*Brevoortia tyrannus*) in Chesapeake Bay on the east coast of the USA. The virus isolated from the brain of diseased fish had the typical biological, biophysical and biochemical properties of IPNV. Schutz (1984) isolated virus from diseased fry of striped bass (*Morone saxatilis*). The fry displayed the typical signs of IPN, with heavy mortalities.

Viruses similar or identical to IPNV have been isolated from a variety of marine shellfish from around the coast of Britain and the east coast of Canada (Hill, 1976; Underwood *et al.*, 1977; Dobos *et al.*, 1979). Shellfish yielding virus include tellina (*Tellina tenuis*), flat oyster (*Ostrea edulis*), Pacific oyster (*Crossostrea gigas*), American oyster (*C.virginica*), hard clam (*Patella vulgata*), common periwinkle (*Littorina littorina*), and the shore crab (*Carinus macnas*). Though results of infectivity tests are variable, at least eight of the shellfish isolates appeared to be pathogenic to rainbow trout in which they produced typical signs of IPN disease. Some of the isolates could cause mild lesions in pacific and flat oyster. The above reports demonstrate that IPNV has an extremely wide host range involving salmonids, non-salmonids, and shellfish from marine waters.

1.12 Classification of IPNV

Nicholson and Pochebit (1981) studied the antigenic relationships among 12 representative isolates from North America and Europe by neutralization kinetics (NK) using antisera to VR 299. Though all isolates were neutralized to some degree, the test revealed a distinct antigenic difference between a number of the isolates. Analysis of NK values suggested that the 12 isolates could be divided into a minimum of three serotypes.

Macdonald and Gower (1981) confirmed the existence of the 3 serotypes of IPNV - VR 299, Ab and Sp, based on neutralization kinetics, size of RNA and size and composition of proteins. By a comparitive study of the above characteristics, they concluded that each serotype is unique. In addition, they observed that phenotypic markers such as host range, plaque size and morphology, and inherent temperature sensitivity were also unique for each serotype. The three serotypes (VR 299, Ab and Sp) are all closely related by serum neutralization but diverged in mw of RNAs and protein composition (Hedrick *et al.*, 1983). The existence of three serotypes, VR 299, Ab and Sp was also confirmed by studies conducted by Okamoto *et al.* (1983). Based on 1/r values ($r = \sqrt{r_1 x\ r_2}$) calculated from cross neutralization tests, they classified 10 selected strains of IPNV into 3 serotypes or groups. Group I included all strains isolated from North America, group II strains from France and group III strains from Denmark and eel virus from Japan.

The antigenic relationships among IPNV isolates are complex and there is disagreement concerning the number of antigenic types that exist from cross neutralization tests. Monoclonal antibodies have been succesfully used to analyse the inter-virus relationships among IPNV isolates from different regions. Caswell-Reno *et al.* (1986) compared 14 different isolates of IPNV using monoclonal antibodies produced against the West Buxton isolate (VR 299 type). Reciprocal blocking ELISA and neutralization assays using monoclonal antibodies identified 4 structurally different epitopes on the virion. Western immunoblot analysis demonstrated that one epitope was present on VP2, the large capsid protein (51K), and two others were on the small structural proteins, VP3 (32K) and VP4 (30K). Comparison of reactivity patterns of the five monoclonal antibodies with various IPNV isolates by ELISA and neutralization test demonstrated that the 14 isolates tested from the 3 serotypes represented a minimum of 9 antigenically distinct isolates. Two monoclonal antibodies identified different epitopes that were highly conserved among and largely restricted to, members of the West Buxton serotype. The other two monoclonal antibodies recognised epitopes present only on some members of this serotype, and the fifth defined an epitope that was more widely distributed among the IPNV isolates in general.

Wolski *et al.* (1986), produced monoclonal antibodies to the Sp serotype of IPNV. In ELISA, they found that out of 12 monoclonal antibodies, 10 were broadly reactive against partially purified representitives of 3 IPNV serotypes (VR

299, Ab and Sp) and two were reactive with the Sp serotype alone.

Hill and Way (1988), based on 50% plaque reduction by rabbit antisera demonstrated the existence of six serotypes of IPNV in addition to the already designated 3 serotypes, VR 299, SP and Ab. They are Hecht (He), Tellina (Te), Canada 1 (C1), Canada 2 (C2), Canada 3 (C3) and Jasper (Ja). Subsequently, Caswell-Reno *et al.* (1989) prepared a panel of monoclonal antibodies to develop an enzyme immunodot assay for rapid identification and presumptive serotyping of aquatic birnaviruses. Their assay could demonstrate antigenic differences between C1, C2, and Ja but not between C3 and C2.

Christie *et al.* (1988), from their cross neutralization and SDS-PAGE studies, designated an IPNV isolate from Norway as the N1 serotype. The isolate was neutralized by homologous immune serum, but not by immune sera raised against the major IPNV serotypes VR 299, AB and SP. In their further attempt to characterize the virus, they used a panel of four monoclonal antibodies directed to the N1 serotype and recorded that out of 40 isolates in Norway, 38 reacted with the N1 serotype. Their study also revealed that the N1 serotype is more closely related to Ja and WB IPNV isolates, since N1 serotype specific monoclonal antibodies reacted only with these isolates.

Lipipun *et al.* (1989), using a panel of monoclonal antibodies, investigated the antigenic relationships of aquatic birnaviruses from Asia. Their studies indicated that all of the Asian birnaviruses share a serogroup specific epitope with related viruses from North America and Europe. Six viruses from Taiwan were identical to the European eel virus and differed from the European Ab virus only by a single epitope.

Hsu *et al.* (1989) compared 13 isolates of IPNV from eel and rainbow trout in Taiwan with serotypes AB, Sp and VR 299 by PAGE analysis of their RNA and early polypeptides. All IPNV isolates from eel and rainbow trout were found to be closely related to the AB serotype. They observed that RNA patterns of some IPN virus resembled that of VR 299, but varied in polypeptide pattern.

Classification of IPNV at the genomic level is drawing attention. Recently, Havarstein *et al.* (1990) sequenced RNA segment A of the N1 serotype and compared it with that of the Ja-IPNV isolate. The isolates were found to differ in nucleotide sequence in the internal part of the gene encoding the VP2 polypeptide.

1.13 Chemotherapy of IPN infection

The work on chemotherapy is very meagre. Economan (1972) reported that inclusion of polyvinyl pyrrolidone-iodine in the diet of brook trout suffering from IPN disease reduced mortality by 50%. However, this work was not substantiated by others.

Migus and Dobos (1980) have shown that virazole/ribavarin (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic nucleoside, at a concentration of 10 μg/ml caused a substantial reduction in the yield of IPNV in infected cell cultures. The chemical inhibits viral replication by suppressing RNA synthesis. Studies by Savan and Dobos (1980) have shown that the mortality rate in 6 week old rainbow trout infected with IPNV was reduced only marginally by treatment with a single exposure to virazole. A dose as high as 400 μg/ml of tank water did not result in a marked reduction in the numbers of deaths. Virazole, which is cytostatic, in effect also has its drawbacks; (i) the antiviral activity is reversed once it is withdrawn from water, ii) it does not selectively suppress virus specific RNA, iii) host nucleic acid synthesis is also affected. In view of these drawbacks this drug was not suggested for use in hatcheries.

Kuznar *et al.* (1986) studied the inhibition mechanism of antiviral lysosomotrophic agents such as chloroquine and ammonium chloride on IPNV replication in cell culture. Both agents inhibited viral replication, but ammonium chloride was more effective, decreasing the virus production by 95%.

1.14 Vaccines against IPNV infection

Sano *et al.* (1981) studied the immune response in adult trout against formalin killed concentrated IPNV. The concentrated immunogen ($10^{9.8}$ $TCID_{50}$ /ml) produced a much higher response in rainbow trout than a less concentrated one ($10^{7.8}$ $TCID_{50}$ /ml). The challenge virus was not re-isolated from kidney and spleen of fish having ND_{50} greater than 10^3, but could be reisolated from organs having an ND_{50} less than 10^3. The maximum ND_{50} was reached between 3 to 4 months post immunization. Their data suggest that vertical transmission of the virus can be blocked by vaccinating broodstock 3-4 months prior to spawning.

Dixon and Hill (1983) inactivated IPNV for vaccine use employing formalin and beta propiolactone (BPL). Incubation with 1:200 dilution of formalin at 20°C for 4 d or longer or a 1:200 dilution of BPL a 4°C for 6 d, completely inactivated the virus. Treatment with formalin and BPL caused reduction in antigenicity of virus by 10-15 and 50%, respectively. Thus, virus inactivated with formalin proved to be highly effective in inducing high titers of neutralizing antibody in rainbow trout.

Bootland *et al.* (1986) observed that brook trout fry vaccinated at 8.5 weeks of age by immersion in an inactivated virus vaccine (dose 10^7 to 10^9PFU/ml) were not protected from a later challenge with IPNV. Vaccinated groups suffered higher mortalities than unvaccinated controls, with the greatest loss associated with the highest vaccine dose. A possible explanation for this increased susceptibility after exposure to a high concentration of antigen is that fry, which are not fully immunocompetent, develop immune tolerance rather than immunity.

A protocol for a standard challenge of IPNV in brook trout, *Salvelinus fontinalis* as a model species, was developed by McAllister and Owens (1986). They found that an immersion challenge for 5 h with 10^5 PFU/ml induced higher and more consistent mortality than did hyperosmotic infiltration challenge. The challenge virus should not be sequentially transferred more than five times in cell culture because further transfer reduces the virulence of the virus.

1.15 Objectives of this thesis

IPNV was first detected in lake trout spawners from Cornwall Lake in Northern Alberta in 1984 (Larson, 1985). The isolate was believed to be indigenous to the lake since salmonids have not been stocked in either Northern Alberta or in the adjacent Northwest Territories (Souter *et al.*, 1986). Lake trout from Cornwall Lake were chosen as brood stock for propagation and enhancement of the sport fish stock in water bodies of Alberta. In this regard it was important to know the prevalence of the virus infection in feral lake trout populations and the pathogenicity of the virus to lake trout and to other salmonids.

A more important aspect of the virus was its origin and antigenic relationships to other Canadian IPNV isolates particularly, Arctic char IPNV from Northwest Territories and Jasper IPNV from Jasper National Park, Alberta. Antigenic differences between some of the North American IPNV isolates was demonstrated by Caswell-Reno *et al.* (1986) using monoclonal antibodies. Later, Hill and Way (1988), based on 50% plaque reduction by rabbit antisera, proposed 4 serotypes of IPNV in Canada- Ja, C1, C2 and C3. Among these Canadian serotypes Caswell-Reno *et al.* (1989) demonstrated antigenic differences between Ja, C1, and C2 by epitope analysis using monoclonal antibodies. However, they could not distinguish C2 from C3 (AC-IPNV). In this context it is of interest to determine the antigenic relationship of LT-IPNV to AC IPNV and C2.

The objectives were to determine the prevalence of the virus infection in the feral lake trout of Cornwall Lake, its pathogenicity to lake trout and other salmonids, and its antigenic and molecular relationship to Ja, AC and other Canadian IPNV isolates.

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CHAPTER 2

Incidence and Pathogenicity of IPNV associated with spawning lake trout in Cornwall Lake

2.1 INTRODUCTION

IPNV is the etiological agent of an economically important disease of salmonid fish (Wolf *et al.*, 1960). The virus usually attacks young fry in hatcheries and mortalities can vary, but greater than 90% have been reported. Though pancreatic tissue is considered to be the primary target organ, virus can be isolated from many other organs including intestine, liver, spleen, kidney and gonad. Earlier studies have shown that fish surviving infection could become carriers, perhaps for their life time (Billie and Wolf, 1969 ; Reno *et al.*, 1978; Yamamoto and Kilistoff, 1979). The persistence of virus in carrier populations appears to be continuous with a number of infected cells producing virus (Hedrick *et al.*, 1982). Virus similar to IPNV have been isolated from various other fish species and marine molluscs in North America and Europe (Hill, 1976; McAllister, 1983). More recently, IPNV has been found to infect eel, tilapia, and trout in Asia (Hedrick *et al.*, 1983; Chen *et al.*, 1985). The number of IPNV isolates from different hosts and geographical areas is increasing.

Lake trout IPNV (LT-IPNV) was first isolated in 1984 from spawning lake trout in Cornwall Lake, an isolated region in Northern Alberta (Larson, 1985). The virus appeared to be indigenous to Cornwall Lake, as cultivable salmonids were not known to have been introduced to this remote region (Souter *et al.*, 1986). Lake trout from Cornwall Lake, were used as brood stock for propagation of fingerlings in Alberta hatcheries from 1982 to 1987, since it is a popular game fish in Canada. In this regard, it is important to know the extent of IPNV infection in the feral lake trout population in Cornwall Lake and the pathogenicity of the virus to young lake trout and other cultivable salmonids. This chapter reports results of studies on the prevalence of the virus in the adult lake trout population of Cornwall Lake and its pathogenicity to lake trout and other cultivable salmonids.

2.2 MATERIALS AND METHODS

Estimation of IPNV carrier rate in adult lake trout population of Cornwall Lake

A total of 27 spawning lake trout of both sexes were selected from a gill net catch from Cornwall Lake during September, 1987. Pyloric caeca, intestine, liver, kidney, gonad and blood were collected aseptically in vials at the site of fish catch and brought to the Edmonton laboratory in ice. The organs were assayed for virus within 48 h after collection.

Approximately 1 g of tissue from the organs were homogenized in 3 ml of MEM-HEPES in a Stomacher Lab Blender (Seward Laboratories, England). Supernatant of tissue extract was obtained by centrifugation at 2000 rpm for 15 min, and 100 μl inoculated to CHSE-214 cells monolayer in 96 well microtiter plates. CHSE-214 cells were grown in MEM-HEPES with 5% fetal bovine serum, 200 I.U. of penicillin/ml and 200 μg/ml of streptomycin. Cytopathic effect (CPE) was observed over a period of 14 d and the virus infectivity titer ($TCID_{50}$) determined according to the method of Karber (1931). Doubtful CPE was confirmed by repassing the contents to new CHSE-214 cell monolayers.

Leucocytes were assayed for virus by co-cultivating with CHSE-214 cells according to Yu *et al.* (1982). Blood collected in heparinized vials was diluted with an equal quantity of fetal bovine serum, layered on Histopaque solution (Sigma) and centrifuged at 700 rpm for 20 min at 4^{o}C. Leucocytes pelleted as buffy coats were separated and suspended in 1 ml HBS. Leucocytes in 0.1 ml suspension were added to each well of a 24 well plate having CHSE-214 cell monolayer and co-cultivated. CPE was observed over a period of 14 d.

Experimental infection of rainbow trout with LT-IPNV

Rainbow trout fry procured from Sam Livingston Hatchery, Calgary, were maintained in the laboratory for a week prior to experimental infection. Fifty, five week old fry were inoculated in duplicate by immersion for one h in one liter of water containing LT-IPNV (second passage in CHSE-214 cells) at 10^5 $TCID_{50}$/ml. After infection, fishes were maintained in two liter plastic containers with running water at 10-12°C. An uninoculated control batch consisting of 50 fry was mainained under similar conditions as above for comparison. Fry that died up to 22 d post inoculation (DPI) were assayed for the virus. Those surviving the infection were sampled at 10 d intervals up to 102 DPI to determine virus persistence in the survivors.

Whole fish were homogenized in 3 ml MEM-HEPES in a Stomacher Lab Blender. The homogenate was centrifuged at 2000 rpm for 15 min and 100 μl inoculated to CHSE-214 cell monolayer in 96 well plates. CPE was observed for 14 d and virus quantitated by the limiting dilution method of Karber (1931).

Experimental infection of brook trout with LT-IPNV

Brook trout fry procured from Sam Livingston Hatchery, Calgary, were maintained in the laboratory for one week prior to experimental infection. Fifty, five week old fry were inoculated in duplicate with LT-IPNV (second passage in CHSE-214 cells) at 10^5 $TCID_{50}$/ml and maintained as described for rainbow trout. An uninoculated control batch of 50 fry were maintained under similar conditions. Fish mortalities were assayed for virus as described in the previous procedure.

To study the course of LT-IPNV infection in brook trout, 3 month old fry were infected with LT-IPNV at 10^5 $TCID_{50}$/ml . Four fish were sampled at 1, 2, 4, 8 and 16 h post infection during first day and on alternate days until 27 DPI. Half of the samples were assayed whole, and the remaining half were assayed by organs. Organs including gill, heart, pyloric caeca, liver, intestine and kidney were separated aseptically under the dissecting microscope, homogenized in 1 ml MEM-HEPES and assayed for virus as described before.

Experimental infection of lake trout with LT-IPNV

Lake trout fry from two different sources were used for experimental infection. First, 25 three and a half month old lake trout fry procured from Cold Lake Hatchery, Cold Lake, Alberta, were inoculated with LT-IPNV (second passage in CHSE-214 cells) by immersion at 10^5 $TCID_{50}$/ml. A control uninoculated group of 25 fry were maintained for comparison. Fish were maintained at 10-11°C and observed for mortality for 30 d.

The experiment was repeated with Slate Island strain lake trout fry obtained from Fish Culture Station, Dorion, Ontario. One day old hatchlings were transported to the Edmonton laboratory and reared until used for experimental infection. Fifty, 8 week old fry were infected in duplicate with LT-IPNV as described before and maintained with an uninoculted batch of 50 fry for comparison of mortality.

To study the course of LT-IPNV infection in lake trout, six week old fry were infected with LT-IPNV by immersion at 10^5 $TCID_{50}$/ml. Three fry each were sampled at 0, 2, 4, 8 and 16 h post infection during the first day and on alternate days to 29 DPI. Whole fry were assayed for virus by the limiting dilution method.

2.3 RESULTS

Prevalence of IPNV infection in spawning lake trout population in Cornwall Lake

To determine the IPNV carrier rate, lake trout spawners were sampled and assayed for virus. Fig. 2.1 shows the quantity of IPNV in organs of the spawning lake trout. Of 27 fish sampled, 12 (44.4%) were carrying the virus. Among the various organs assayed- gills, liver, kidney, pyloric caeca, intestine and gonad, only the pyloric caeca and intestine showed the presence of virus. Leucocytes were negative for virus. Virus titers in intestine and caeca ranged from 3.1 to 7.25 log $TCID_{50}$/g of tissue. All positive fish had virus in their pyloric caeca, while only 50 percent of the positive fish had virus in the intestine. Both males and females were found to carry the virus in the pyloric caeca and intestine.

Pathogenicity of LT-IPNV to fry of salmonids in experimental infection

a. Pathogenicity to rainbow trout

There were few mortalities in rainbow trout experimentally inoculated with LT-IPNV. The mortality in the infected lot was 10% compared to 4% in the uninfected control lot. Assay of dead fish from the infected lot showed virus titer ranging from 3.9 to 7.4 log $TCID_{50}$/g of fish (Fig.2.2). Virus was not detected in fish mortalities in the control lot.

Since mortalities were low, surviving fish were sampled (Fig. 2.2) at 10 d intervals to 102 DPI. Surviving fish assayed from 22 to 102 d contained virus ranging from 3.6 to 4.95 log $TCID_{50}$/g of fish. In all, 82% of surviving fish that were assayed carried the virus.

b. Pathogenicity to brook trout

Brook trout are known to be more susceptible to IPNV infection than are

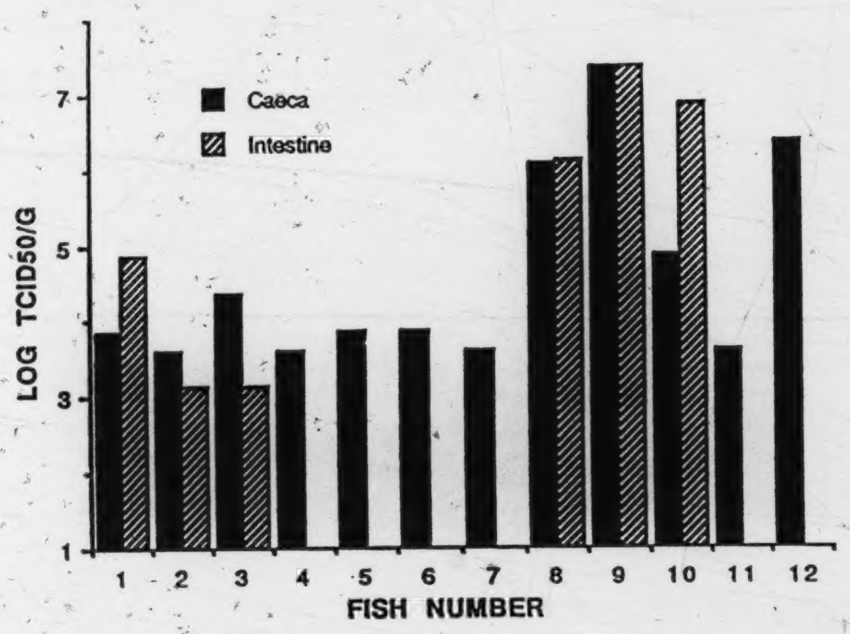

Fig. 2.1. Quantitation of IPN virus in pyloric caeca and intestine of spawning lake trout collected from Cornwall Lake. Approximately 1 g of tissue was removed aseptically and assayed for infectivity. The limiting dilution assay and the $TCID_{50}$ was calculated according to the method of Karber (1931).

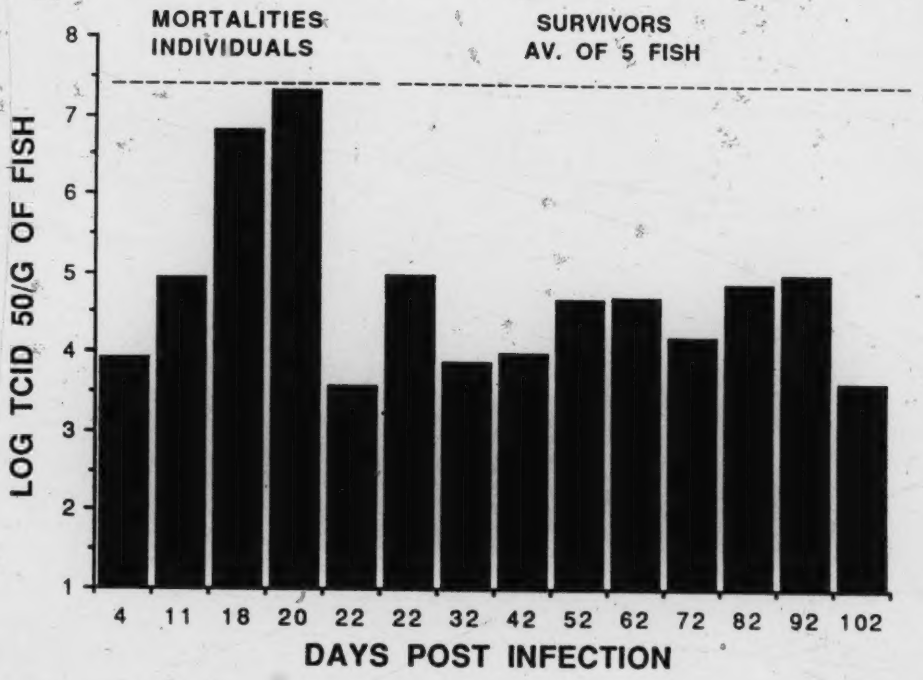

Fig. 2.2. Virus titer in rainbow trout experimentally infected with LT-IPNV. Fifty, five week old rainbow trout were infected by immersion in water containing LT-IPNV at 10^5 $TCID_{50}$/ml for 1 h. Fish that died up to the 22nd DPI were collected daily and the remaining survivors were sampled at 10 d intervals to 102 DPI. Whole fish were homogenized and assayed for virus by the limiting dilution assay.

other salmonid fishes. Fig. 2.3 shows cumulative mortality and Fig. 2.4 virus titer in the 5 week old brook trout fry experimentally infected with LT-IPNV. Fry exhibited typical signs of IPN disease, i.e, black body colour, swollen belly and whirling movements. Mortality increased steadily, from 10 DPI reaching 74% during the experimental period of 30 d. In the uninfected control lot, mortality was 16%. More than 90% of fish that died in the infected lot showed virus titers greater than 3.5 log $TCID_{50}$/g of fish. Mortalities in the control lot did not have any virus.

Figs. 2.5 and 2.6 show the course of LT-IPNV infection in experimentally infected 3 month-old brook trout. Virus titer started increasing from 3 DPI, and from then on ranged between 3.5 to 7.25 log $TCID_{50}$/g to 27 d. The specific location of virus replication was determined by assaying the various organs separately (Fig. 2.6). The intestine and caeca showed virus 1 DPI at titers more than 5 log $TCID_{50}$/g tissue. Virus in the gills was detected from 2 DPI whereas virus the in heart and liver was not detected until 10 DPI. Virus was consistently detected in all of the organs from 11 DPI at titers more than 5 log $TCID_{50}$/g of tissue. Highest virus titers in the organs were obtained from 13 and 20 DPI, the period when mortalities were high.

c. Pathogenicity to lake trout

Experimental LT-IPNV infection did not cause any mortalities in three and a half month old lake trout fry. These fry were propagated from eggs of Cornwall Lake lake trout at the Cold Lake Hatchery. This lack of pathogenicity was also noticed in the experimental infection of 8-week old Slate Island strain lake trout procured from Ontario. The cumulative mortality in the infected lot was 12% compared to 10% in the uninfected control. However, dead fish from experimentally infected lot had virus ranging from 3.67 to 4.14 log $TCID_{50}$/g of

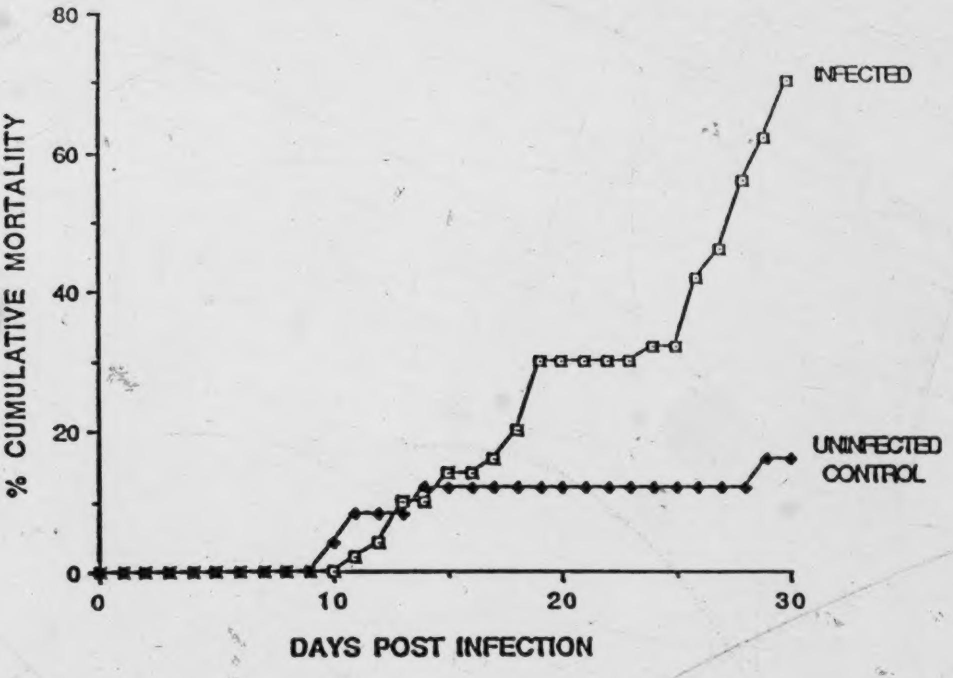

Fig. 2.3. Mortality of brook trout fry experimentally infected with LT-IPNV. Five week old brook trout fry were infected by immersion in water containing 10^5 $TCID_{50}$/ml of LT-IPNV for 1 h. Mortalities in the control and infected lots were recorded for 30 d.

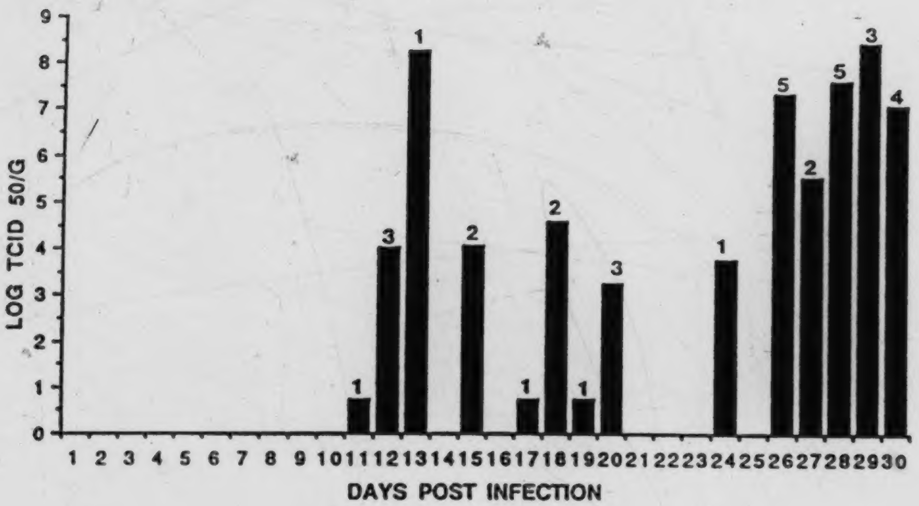

Fig. 2.4. Virus titer in five week old brook trout fry experimentally infected with LT-IPNV. The fish were infected by immersion for 1 h in 1 litre of water containing 10^5 $TCID_{50}$/ml of LT-IPNV. Dead fry collected for 30 d were assayed for virus by the limiting dilution method. Numbers on the top of bars indicate the number of IPNV positive fish.

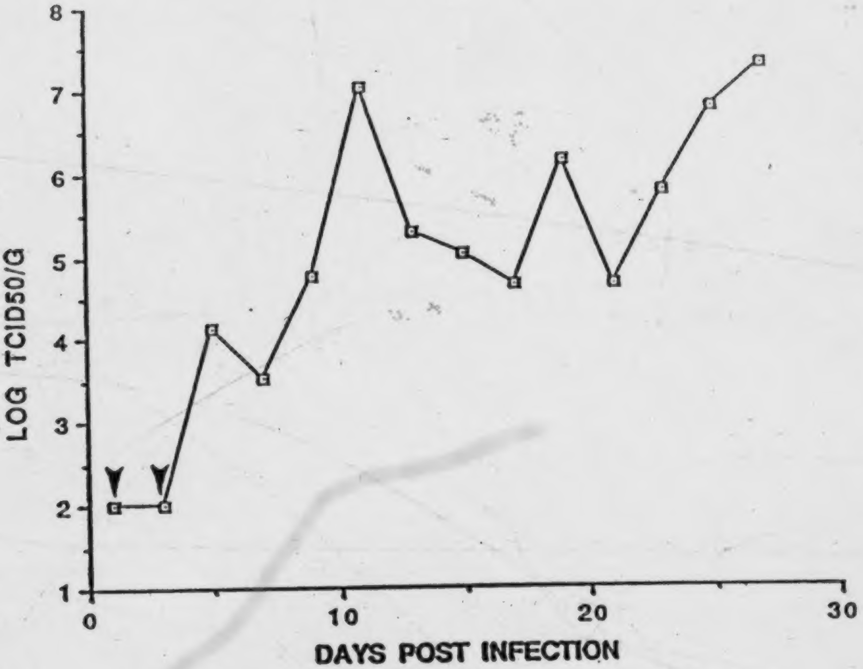

Fig. 2.5. Virus titer in brook trout fry experimentally infected with LT-IPNV. Three month old brook trout fry were infected by immersion in water containing 10^5 $TCID_{50}$/ml of LT-IPNV for 1 h. Three fish were sampled on alternate days for 29 d and whole fish was assayed for virus by the limiting dilution method. Arrows point less than 2 log, the minimum $TCID_{50}$/g the assay could detect.

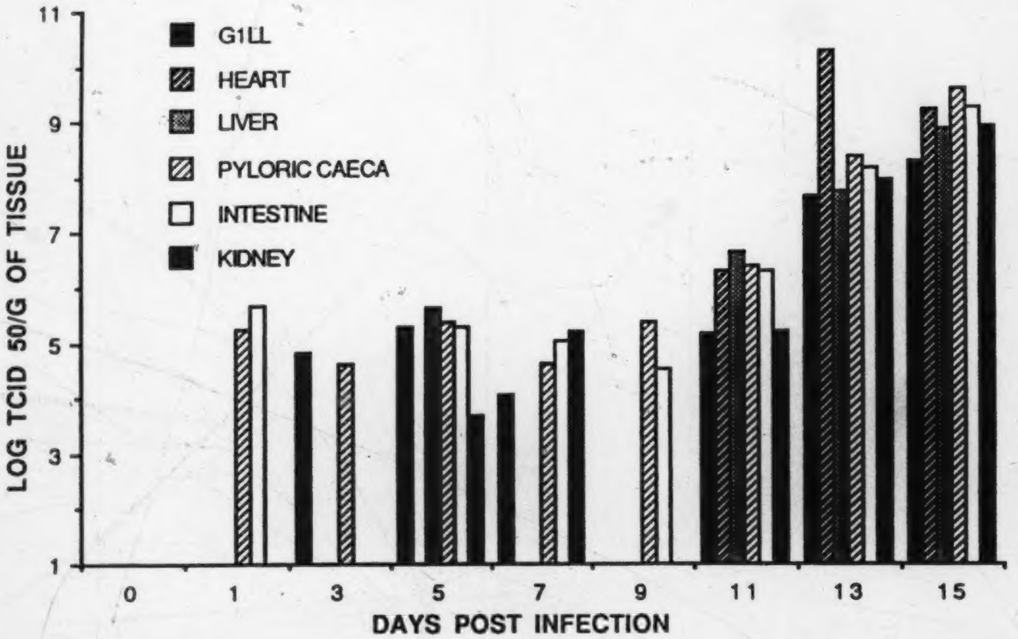

Fig. 2.6. LT-IPNV titers in the organs of experimentally infected brook trout fry. Three month old brook trout fry were experimentaly infected with LT-IPNV at 10^5 $TCID_{50}$/ml for 1 h and sampled on alternate days for 15 d. Organs from individual fry were separated aseptically under the dissecting microscope and assayed for infectivity.

tissue.

Fig. 2.7 shows the course of LT-IPNV replication in six week old lake trout fry. The virus titer which began to increase from 2 DPI ranged from 3.72 to 4.4 log $TCID_{50}$/g during a period of 29 d.

2.4 DISCUSSION AND CONCLUSION

Our studies on the prevalence of IPNV revealed that 44.4% of adult lake trout in Cornwall Lake are infected with the virus. Similar studies conducted by the Veterinary Service laboratory, Government of Alberta, Edmonton, also indicated that 40% of the lake trout population of Cornwall Lake carry the virus (B. Larson, personal communication). It is interesting to note that 42.8% of the Arctic char population in the Mackenzie River Delta are infected with IPNV (Souter *et al.*, 1986).

Among the various organs assayed, virus was detected only in the pyloric caeca and less frequently in the intestine. In contrast, in Arctic char the virus was detected most frequently in the kidney (49%) and the caeca (43%) and to a lesser extent in spleen, liver and gonad (Yamamoto, 1989). In most studies, kidney tissue of carrier fish has been shown to harbour IPNV consistently (Yamamoto, 1978). From these results on the natural infection of lake trout, it appears that the LT-IPNV differs from that of Arctic char in the organ of persistence.

LT-IPNV was highly pathogenic to brook trout fry causing a mortality of 74% in 30 d. Typical IPN symptoms including dark body, swollen belly and corkscrewing movements were observed in brook trout fry infected with LT-IPNV. Mortalities were noticed from 10 DPI. Pyloric caeca and intestine were found to be the early sites of viral replication. The virus was detected in these organs at $TCID_{50}$ greater than 5 log/g of tissue one day after infection. Gills also appeared to be involved in

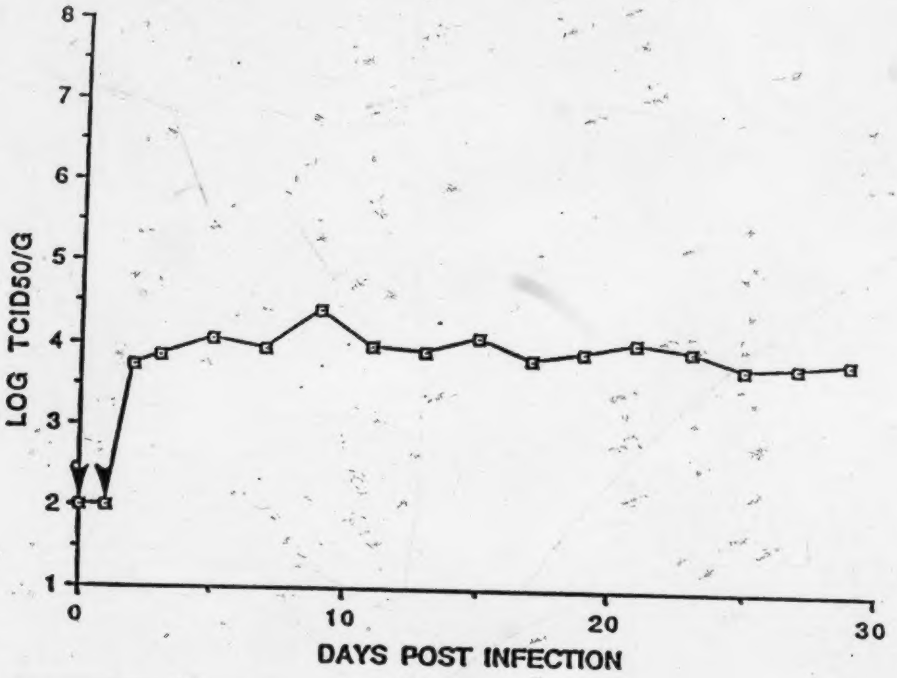

Fig. 2.7. Virus titre in lake trout fry experimentaly infected with LT-IPNV. Six week old fry were infected by immersion in water containing 10^5 $TCID_{50}$/ml of the virus for 1 h. Fry were sampled at 2, 4 and 8 h on the first day and on alternate days for 29 d. Whole fry were assayed for virus by the limiting dilution method. Arrows point less than 2 log, the minimum the assay could detect.

early replication of the virus in which virus titers more than 4.5 logs/g tissue was recorded from 2 DPI. The virus was less pathogenic to rainbow trout but it persisted in them for a period of upto 102 d. The virus was more pathogenic to brook trout compared to rainbow trout, which supports the earlier findings (Hill, 1982; McAllister, 1983) that brook trout are more susceptible than rainbow trout to IPNV infection.

The virus was not pathogenic to young lake trout, it's natural host. The virus did not cause mortality in lake trout of two different age groups obtained from two different hatcheries namely Cold Lake Hatchery, Cold Lake, Alberta and Fish Culture Station, Dorion, Ontario.

As observed in rainbow trout, the virus persisted in the young infected lake trout and hence may persist into adulthood. In the adult lake trout the virus appears to persist only in intestine and pyloric caeca. It appears that lake trout IPN virus is non-pathogenic to its natural host but low to highly pathogenic to cultivable salmonid species.

AC-IPNV differs from LT-IPNV in its pathogenicity. AC-IPNV caused 50% mortality in the Arctic char, 35% in brook trout (Yamamoto, 1989). However, both IPNV isolates have one characteristic in common; they caused mortality in brook trout fry begining 10 DPI. After 10 DPI the virus was detected at titers higher than 10^5 $TCID_{50}$/g tissue. Normally, IPNV causes mortality from 5 to 6 DPI when virus titers reach more than 10^5 $TCID_{50}$/g tissue (McAllister, 1983; Okamoto *et al.*, 1984).

According to Souter *et al.* (1986) there is no farming or seeding of salmonids in the Northwest Territories. Therefore, it appears that LT-IPNV, like AC-IPNV, is indigenous to the Cornwall Lake region. The LT-IPNV appears to be different from AC-IPNV with respect to carrier state in that different organs of the host are affected.

From the above studies it can be concluded that 44.4% of adult lake trout in Cornwall Lake are infected with IPNV. The virus persists mainly in pyloric caeca and is non-pathogenic to its natural host but pathogenic to other cultivable salmonids. The virus differs from AC-IPNV with respect to site of persistence and pathogenicity. Further studies should be carried out on the antigenic relationship of the virus to other Canadian IPNV isolates.

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CHAPTER 3

Antigenic Characterization of LT-IPNV from Cornwall Lake

3.1 INTRODUCTION

IPN virus causes serious disease in hatchery reared salmonids. Viruses similar or identical to IPNV have been isolated from various salmonid and non salmonid fish species and marine molluscs from all over the world (Hill, 1976; McAllister, 1983 ; Hedrick *et al.*, 1983 ; Chen *et al.*, 1985). Serological studies of aquatic birnaviruses indicate that most cross react to some extent, but significant antigenic differences exist among numerous isolates (Caswell-Reno *et al.*,1986). Based on the relative degree of cross neutralization by rabbit antisera, three main serotypes namely VR 299 (North America), Sp and Ab (Europe) were designated (Hill, 1976; Macdonald and Gower, 1981; Okamoto *et al.*, 1983). Later, Hill and Way (1983) examined 175 virus isolates from 44 fish and shellfish from 11 countries by cross neutralization with rabbit antisera. Their findings indicated that there are six additional serotypes. Further investigation of IPNV neutralization kinetics with rabbit antisera, showed significant differences among individual members of the VR 299 serotype (Nicholson and Pochebit, 1981). Subsequently, Caswell-Reno *et al.* (1986), using monoclonal antibodies produced against West Buxton-IPNV, demonstrated antigenic differences among isolates grouped under the VR 299 serotype.

Lake trout IPNV was first detected in 1984 (Larson, 1985) in tissues of spawning lake trout in Cornwall Lake, an isolated region in Northern Alberta (Fig. 3.1). Two other IPNV isolated earlier in Western Canada were Ja-IPNV from brook and rainbow trout in Jasper, Alberta and AC-IPNV from Arctic char in the Northwest Territories. AC-IPNV appears to be present in the Arctic char

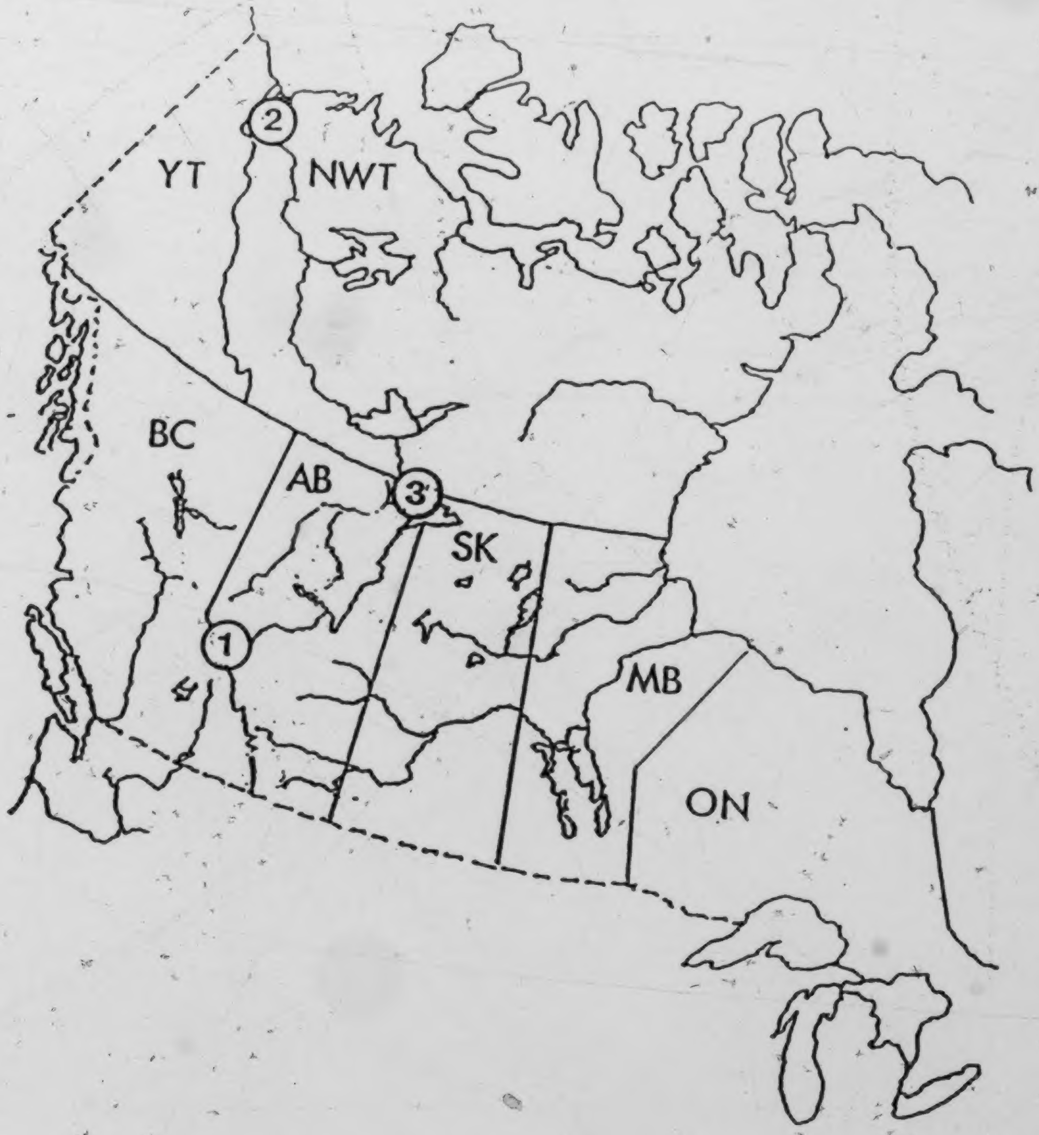


Fig. 3.1. Map of Western Canada showing the geographical distribution of IPNV isolations. 1. Ja-IPNV from Jasper National Park in Alberta; 2. AC-IPNV from Western Mackenzie River Delta in Northwest Territory; 3. LT-IPNV from Cornwall Lake in Northern Alberta

populations in the western region of Mackenzie Delta region and absent in the eastern region. Hill and Way (1988) have proposed that IPNV in Canada be placed in 4 separate serotypes based on 50% plaque reduction by rabbit antisera. The serotypes were designated Canada 1 (C1) from Atlantic Salmon, Canada 2 (C2) from trout of Eastern Canada, Canada 3 (C3) from Arctic char, and Jasper (Ja) from trout in Jasper, Alberta. In this serotyping, IPNV from lake trout in Cornwall Lake was not included. A recent serotyping by immunodot assay using monoclonal antibodies (Caswell-Reno *et al.*, 1989), demonstrated that C1, C2, and Ja are distinct serotypes. However, from their study it was not clear whether C3 is a distinct serotype from C2. LT- and AC-IPNV were isolated from adult carriers from two different remote regions for which there are no records of salmonid introduction.

Therefore, it is important to investigate the serotype of LT-IPNV, in comparison to other Canadian IPNV isolates, particularly Ja and AC-IPNV (C3). This chapter reports results of epitope analysis of LT-IPNV in comparison to other Canadian isolates using polyclonal and monoclonal antibodies. In addition, the polypeptide composition of the virus was analysed and compared to that of other isolates.

3.2 MATERIALS AND METHODS

Virus stocks and their purification

LT-IPNV from Cornwall Lake, AC-IPNV from Northwest Territories, Ja-IPNV from Jasper Hatchery and VR 299 (ATCC) isolates were available in our laboratory. Isolates of Canada 1 (C1) from Atlantic salmon and Canada 2 (C2) from trouts of Eastern Canada were provided by Dr. R. K. Kelly, Freshwater Fisheries Research Center, Winnipeg, Manitoba. The BC-1 IPNV isolate from British Columbia was provided by Dr. Trevor Evelyn, Department of Fisheries and

Oceans, Nanaimo, British Columbia. All isolates were plaque purified thrice and propagated in CHSE-214 cells according to Lannon *et al.* (1984).

Virus isolates for immunization and SDS-polyacrylamide gel eletrophoresis were purified by CsCl centrifugation according to Chang *et al.* (1978). Cell associated virus was extracted with Freon and pooled with virus precipitated from the cell culture supernatant using polyethylene glycol (mw 20,000). Pooled virus samples were layered onto 20, 30 and 40% CsCl (w/v) gradients and centrifuged at 98,000X *g* for 16 to 24 h. The virus bands collected were dialysed against TNE buffer (0.025 M Tris, 0.1 M NaCl, 1 mM EDTA pH 7.3). The virus preparation was examined by observing negatively stained samples in the electron microscope.

For coating immunolon I plates used in ELISA, LT-IPNV was purified by sucrose gradient centrifugation (Caswell-Reno *et al.*, 1986). Freon extracted and PEG precipitated virus was partially purified by centrifugation at 98,000X *g* for 3 to 4 h in a 30 to 60% sucrose gradient. The virus band obtained was resuspended in TNE buffer and pelleted by centrifugation at 30,000 rpm for 4 h. The virus pellet was resuspended in 0.01 M PBS buffer for use in ELISA.

Analysis of IPNV polypeptides

IPN virus isolates were purified in CsCl according to Chang *et al.* (1978). The protein concentration of purified virus preparations was estimated using the BioRad protein assay kit. Using crystalline BSA as a standard, readings were obtained colorimetrically in a Pye-Unicam SP-8 UV/VIS spectrophotometer at 595 nm. Virus preparations containing 40 μg of each isolate (10^{10} $TCID_{50}$/ml) in TNE buffer, were mixed with an equal quantity of sample buffer containing 5% 2-mercaptoethanol and 2% SDS. The mixture was heated at 95°C for 2-3 min and loaded on 10% SDS-polyacrylamide gels (Laemmli, 1970). Samples were electrophoresed at 100 volts for approximately 90 min in a 14.4 mM Tris-glycine running buffer pH 8.8 using an MVG 10 miniature vertical gel system (Tyler Research Institute, Edmonton). After electophoresis, the gel was stained with Coomassie blue for 45 min and photographed after destaining. The experiment was repeated by electophoresces of samples in a longer separating gel (14cm) for 6 h, in order to get better resolution of polypeptides.

In another SDS-PAGE gel, polypeptide bands of LT and AC-IPNV were scanned after through destaining using a Chromoscan-3 densitometer. Percentage composition of each polypeptide band was estimated from the integral area of the corresponding peaks on the scan.

Preparation of rabbit antiserum to LT-IPNV

LT-IPNV purified in CsCl and dialysed in TNE buffer ($10^{10.2}$ $TCID_{50}$/ml) was mixed with an equal volume of Freund's complete adjuvant. Rabbits were administered 1 ml of the above mixture 2 times, at 15 d intervals by intramuscular injection. The animals were bled by cardiac puncture 30 d after the first injection. Blood was clotted at 4°C for 2 h, and the serum separated by centrifugation at 1000X *g* for 20 min at 4°C

Antisera to AC and Ja-IPNV isolates which were made similarly were available in the laboratory.

Cross neutralization test

Aliquots from serial doubling dilutions of antiserum were mixed with equal volumes of $10^{2.2}$ $TCID_{50}$/0.1 ml of virus for 1 h at room temperature. One tenth of one ml of the mixture was inoculated into each well of a CHSE-214 cell monolayer in a 96 well microtiter plate and observed for 14 d. The experiment was carried out in duplicate and neutralizing antibody titers (ND_{50}/0.1 ml) are expressed as the reciprocal of the highest dilution of antiserum protecting 50% of inoculated cell cultures (Karber,1931). The serological relationships 1/r among the three viruses were calculated from the formula $r=\sqrt{r_1 \times r_2}$, where r_1 and r_2 are the titer ratios (heterologous titer divided by homologous titer for respective antisera) (Archetti and Horsfal, 1950).

Production of hybridomas producing LT-IPNV antibodies

Eight week old female Balb/C mice were injected intraperitoneally with 0.06 ml of CsCl purified LT-IPNV ($10^{10.5}$ $TCID_{50}$/ml) in 0.01 M PBS buffer. A second dose of 0.15 ml of purified virus was given (IP) on the 21st d, followed by a third dose of 0.15 ml (IV) on the 28th d. Three days later, mice were bled and the LT-IPNV antibody titer in the serum was measured by ELISA. Spleens from mice having higher titers of antibody in ELISA (>5000 dil of antisera) were used for fusion on the 32nd d.

Hybridoma cells were produced according to Winton *et al.* (1988). Spleen cells were suspended in serum free RPMI medium (Gibco) kept at 37°C, and mixed with 2 d old actively growing SP2/o myeloma cells at an approximate ratio of 1:10. The cell mixture was co-pelleted by centrifuging at 2000 rpm for 10 min and fused by slowly mixing the pellet for 10 min in the presence of a 1:1 mixture of RPMI medium and polyethylene glycol (80% PEG mw 1500 and 20% PEG mw 4000). The final volume of the mixture was brought up to 50 ml with serum free RPMI medium and the cell mixture was pelleted at 500-1000 rpm for 10 min. The pellet was slowly triturated in the presence of 5 ml of RPMI HAT (hypoxanthine 2.5x 10^{-5} M, aminopterin 10^{-7} M, and thymidine 4x10^{-6} M) and final volume increased to 200 ml with RPMI-HAT medium containing 15% fetal bovine serum, 50 units of penicillin and streptomycin (Sigma Chemicals). The above suspension was transferred to 96 well microtiter plates at 2 drops per well. A control plate consisting of SP2/o myeloma cells alone in RPMI-HAT medium was maintained to compare the succes of fusion. Fused cells were maintained at 37^{o}C with 5% CO2 and 80% relative humidity in a Steri-cult incubator. Five days post fusion cells were fed with 2 drops of RPMI-HAT medium per well. RPMI-HAT medium was replaced by RPMI-HT medium (hypoxanthin 2.5x10^{-5} M, thymidine 4x10^{-6}M) in all wells on the 8th d, followed by feeding with RPMI-HT on the 11th d. From the 14th d visible hybridomas were marked and the supernatant assayed for LT-IPNV antibodies by ELISA on alternate days until 25th d post fusion.

Hybridomas producing antibodies to LT-IPNV were transfered to 24 well plates, grown for 3 to 4 d, and the supernatant then subjected to preliminary isotyping. At this stage an aliquot of positive hybridoma cells was frozen at -80^{o}C for future use. Cells producing IgG and mixtures of IgG and IgM isotypes were then screened against CHSE-214 cells antigen in ELISA. The antigen was prepared by freeze thawing of CHSE-214 cell monolayer, followed by centrifugation. The pellet was resuspended in carbonate-bicarbonate buffer (pH 9.4), coated on to Immunolon I plate and ELISA carried out with hybridoma culture supernatant. Hybridoma producing antibody to CHSE-214 cell antigen were then eliminated. LT-IPNV specific hybridomas were then minicloned thrice on spleen feeder cells. In each minicloning step, the hybridoma cells were diluted from 10^{-1} to 10^{-5} dilutions and clones were selected from the highest dilution. Spleen feeder cells were prepared from spleens of unimmunized mice. The cells were suspended in serum free RPMI medium and plated on to 96 well microtiter plate, and used as a feeder layer immediately or after storing at -80^{o}C.

After minicloning thrice, stable antibody producing clones were grown in 24 well plate and supernatants isotyped again using mouse monoclonal sub isotyping kit (Hyclone laboratories, Logan, Utah, USA). Initial microneutralization assay was performed with supernatant from all the selected clones. Equal quantities of LT-IPNV ($10^{2.2}$/ 0.1ml $TCID_{50}$) and 4 d old hybridoma cell culture supernatant were mixed for 1 h at room temperature. One tenth of one ml of the above mixture was inoculated to each well of CHSE-214 cells monolayer and cytopathic effect observed for 14 d. Culture supernatants with no CPE were considered as neutralization positive.

Detection of LT-IPNV antibody producing hybridomas by enzyme linked immunosorbent assay (ELISA)

LT-IPNV partially purified on sucrose gradient was diluted in carbonate-bicarbonate buffer (pH 9.2) to 10^8 $TCID_{50}$/ml and coated to Immunolon I plate (Dynatech Laboratories, USA) for 20 h at room temperature. After washing the wells with 0.01 M PBS containing 0.05% Tween-20, non-specific binding sites were blocked by adding 5% skimmed non-instant milk (Nu-maid ,Edmonton,Alberta) for 1 h. Later, wells were washed with PBS-Tween and 100 μl of hybridoma supernatant was added to each well and incubated for 1 h. After washing the plates thrice with PBS-Tween, 100 μl of 1/2000 IgG-Peroxidase (Calbiochem, La Jolla, CA) in PBS-Tween was added to each well and incubated for 1 h. Later,the substrate consisting of 4 mg of O-Phenyl diamine dihydrochloride (Sigma Chemicals) and 4 μl of 30% H_2O_2 in 10 ml phospate citrate buffer (pH 5.2) was added. Color development was stopped in 20 min by addition of 0.1 N HCl. Absorbance was measured at 490 nm in a Titertek Multiskan RMC ELISA reader. Supernatant of hybridomas giving absorbance more than 0.1 (background absorbance) was selected as a positive reading.

Production of LT-IPNV MAbs *in vitro*

Hybridoma cells were grown in RPMI medium with 5% fetal bovine serum in the absence of antibiotics. After 4-5 d, culture supernatants were centrifuged at 4000 rpm and clear supernatants were reinoculated with fresh hybridoma cells. Antibody rich supernatant was finally harvested after the second culture and their antibody titer determined by ELISA. LT-IPNV was coated to immunolon-I plate at $10^{8.5}$ $TCID_{50}$/ml in carbonate and bicarbonate buffer, pH 9.6 and incubated with serial two fold dilution of hybridoma culture supernatant in triplicate. ELISA was carried out as described before. Dilution of culture supernatants giving ELISA absorbance of approximately 1.5 at the above antigen concentration was used in Western blot, immunodot and neutralization assays.

Production of LT-IPNV MAbs *in vivo*

Eighteen month old male Balb/C mice were primed with pristane (Aldrich) at 0.5 ml per mouse (IP). Ten days later, actively growing hybridoma cells were injected (IP) at 10^6 to 10^7 cells per mouse in 0.5 ml PBS buffer. Ascites developed in mice were harvested from 8 to 10 d after injecting cells. Ascites were cleared of clots by centrifuging at 10,000 rpm, and stored at -20°C for further use.

Determination of epitope specificity of LT-IPNV MAbs by reciprocal blocking ELISA

Epitope specificity was determined by the method of Friguet *et al.* (1983). First an optimum concentration of LT-IPNV which can be easily saturated with its monoclonal antibodies was determined. For this purpose LT-IPNV partially purified in sucrose gradients was coated (100 μl/well) on to Immunolon I plates at

$10^{7.5}$, $10^{7.75}$, $10^{7.90}$ and $10^{8.0}$ $TCID_{50}$/ml in carbonate bicarbonate buffer (pH 9.4). Four ascites were chosen at random, and their serial two fold dilutions were incubated to saturate the coated antigen. ELISA was conducted as described before and absorbance with each ascites dilution was measured. Of the above antigen concentrations, $10^{7.5}$ $TCID_{50}$/ml was found to be optimum for saturation with lower concentrations of ascites.

LT-IPNV was coated to Immunolon I plate at 100 µl/well ($10^{7.5}$ $TCID_{50}$/ml) for 20 h at room temperature, and non-specific sites were blocked by adding 1% BSA in PBS containing 0.05% Tween-20 for 45 min. The wells were washed once with PBS-Tween and incubated with serial two-fold dilution of ascites for 1 h. After washing 3X with PBS-Tween, IgG-HRP at 1:2000 dilution in PBS-Tween was added and incubated for 1 h. Substrate (4 mg O-Phenyl diamine dihydrochloride, 4 µl of 30% H_2O_2 in 10 ml phosphate citrate buffer PH 5.0) was added at 100 µl/well. Colour was allowed to develop for 20 min and was stopped by adding 0.1 N HCl. Absorbance was measured at 490 nm. Saturation curves were obtained by plotting absorbance against ascites dilution. The dilution of ascites on the saturation curve at which plateau starts was considered as the saturation point. ELISA absorbance were also measured by increasing the IgG-HRP concentration by two fold, keeping the antigen and antibody concentration constant. This was to ensure that antigen saturation is achieved due to increased concentration of antibody, and not due to exhaustion of conjugate.

Immunolon I microtiter plates were coated with 100 µl /well of LT-IPNV ($10^{7.5}$ $TCID_{50}$/ml) in coating buffer (0.5 M carbonate bicarbonate buffer, PH 9.4) at room temperature for 20 h. The plates were washed and non-specific binding sites were blocked by adding 1% BSA in PBS-Tween 20 for 45 min. After washing, antigen was incubated with ascites of the first MAb (MAb_1) and incubated for 1 h.

After washing 3 X, ascites of the second Mab (Mab_2) was added, incubated, and washed as above. Ascites were used at concentrations 25 to 100 fold more than the antigen saturation point. After incubation with MAb_2, goat anti-mouse IgG-HRP (Calbiochem) at 1/2000 in PBS-Tween was added at 100 μl per well and incubated for 1 h. Finally, substrate was added and incubated for 20 min. The reaction was stopped by adding 0.1 N HCl and absorbance read at 490 nm. A 1:500 dilution of ascitic fluid of a hybridoma producing non-specific antibody to LT-IPNV (LT0) was used as negative control. ELISA absorbance of MAb_1 and MAb_2 at antigen saturation point was determined separately and additivity index (AI) was calculated according to Friguet *et al.*,(1983) as shown below.

$$AI = 100x\ ([(2 \text{ x A of } Mab_1 \text{ and } MAb_2 \text{ together})/(\text{A of } MAb_1 \text{ alone}) + (\text{A of } MAb_2 \text{ alone})] - 1)$$

Reciprocal ELISA was carried out for all of the 28 MAb pairs and AI calculated. Two way analysis of variance (ANOVA) test was applied to determine AI for homologous MAb pairs, at which statistically additivity is zero. MAb pairs giving reciprocal AI values more than zero were considered as reacting with two different epitopes on the LT-IPNV antigen.

Analysis of LT-IPNV MAbs polypeptide specificity by Western blot

LT-IPNV purified in CsCl (10^{10} $TCID_{50}$/ml, approximately 40 μg protein) was treated with sample buffer containing 5% 2-mercaptoethanol and 2% SDS at 90^oC for 3 min. The denatured sample was electrophoresed in 10% polyacrylamide SDS gel (Laemmli, 1970), at 100 volts for approximately 90 min in 14.4 mM Tris-glycine running buffer pH 8.8 using MVG-10 miniature vertical gel apparatus (Tyler Research Institute, Edmonton). Proteins separated were transferred to nitrocellulose paper (Bio-Rad Laboratories) at 100 volts, (250 mA) for 1 h by a Bio-

Rad Mini-Transblot cell apparatus. Lanes of a standard protein marker and virus protein were separated and stained with amido black. Remaining lanes in the paper containing virus protein were cut into 10 mm strips and incubated with blocking solution (3% BSA w/v + 10% FBS in Tris Buffer Saline) for 30 min. The strips were incubated overnight in appropriate dilutions of hybridoma culture supernatants. After thorough rinsing in TBS and blocking as above, strips were incubated with IgG-HRP (Calbiochem) at 1/5000 dilution in TBS buffer for 2 h. The strips were washed in TBS and incubated with substrate (3 mg of 4 chloro-1-naphthol, 3 μl of H_2O_2 per 10 ml of Tris buffer pH 7.6) for 15 min. The strips were washed in distilled water and dried for photography.

Epitope analysis of IPNV isolates using LT-IPNV MAbs by immunodot assay

Immunodot assay was carried out according to the method of Caswell-Reno *et al.* (1989). IPNV culture supernatants (10^8 $TCID_{50}$/ml) were clarified by centrifugation at 1500 rpm for 10 min, and 2 μl from each dotted on nitrocellulose paper (Bio-Rad Laboratories) at room temperature. Two μl of infectious hematopoietic necrosis virus (10^8 $TCID_{50}$/ml) and CHSE-214 cells extract were dotted as negative antigen controls. Nitrocellulose paper was washed 3X with Tris buffer saline (TBS) (pH 7.4) and subjected to non-specific binding for 30 min with 3% bovine serum albumin (w/v) and 10% fetal bovine serum in TBS. Later, the paper was cut into strips and incubated overnight with appropriate dilution of LT-IPNV hybridoma cell culture supernatants. Antibody control consisted of LT-IPNV negative hybridoma culture supernatant. After thorough washing in TBS buffer and subjecting to non-specific binding as above, strips were incubated with 1:500 dilution of IgG-HRP (Calbiochem) in TBS-tween (0.3%) for 2 h. The strips were then incubated in substrate (0.6 mg/ml of 4 chloro-1-naphthol, 1 μl of 30% H_2O_2 per ml of TBS) and purple colour development in 5 min was recorded. The strips were washed in distilled water and dried for photography. The experiment was repeated by increasing concentration of AC, C1, C2, VR-299, Ja and BC1 IPNV isolates by 100 fold.

Epitope analysis of IPNV isolates by neutralization with LT-IPNV MAbs

The concentration of virus isolates was adjusted to $10^{2.2}$ $TCID_{50}/0.1$ ml in MEM-HEPES, and 0.4 ml aliquots mixed with an equal volume of serial two fold dilutions of hybridoma culture supernatant. Hybridoma culture supernatants giving an ELISA absorbance of 1.5 was used for serial dilution. The mixture was thoroughly mixed and incubated for 60 min at room temperature. One tenth of 1 ml from the above mixture was inoculated to CHSE-214 cell monolayer in 96 well microtiter plates. Cytopathic effect was observed for 14 d and the neutralizing antibody titer protecting 50% of inoculated cell culture (ND_{50}) calculated according to Karber (1931).

Immunodot assay of IPNV isolates from Alberta and Manitoba using LT-IPNV specific MAbs

Eight IPNV isolates from various hatcheries and lakes in Alberta were used in the assay. They are, 1) Raven rainbow trout IPNV(89), 2) Charles Lake lake trout IPNV(88), 3) Cold Lake Fish Hatchery RBA-1 (87), 4) Bow River IPNV(89), 5) BP16 SKH(89), 6) Cold Lake Fish Hatchery (89) sentinel Eastern brook trout, 7) white fish IPNV from Cold Lake (89), and 8) Wylie Lake LT-IPNV. In addition, six IPNV isolates from different lake trout of Cornwall Lake were also tested. Grand Rapids and HM were the two isolates from Manitoba. The isolates were grown in CHSE-214 cells and concentrated by 130X by centrifugation at 30,000 rpm for 2 h.

As negative control, 2 μl dots of 100X concentrated Ja, AC, C1, C2 and BC-1 IPNV isolates and CHSE-214 cells extract were used. LT-IPNV served as positve control. Two μl dots of isolates were made on nitrocellulose and the assay carried out as described before using LT-IPNV specific MAbs-LT5 and LT13.

3.3 RESULTS

Analysis of polypeptides of LT-IPNV

Polypeptide analysis of LT-IPNV and other Canadian IPNV isolates - AC, Ja, C1, C2 and BC1 was made by SDS-PAGE (Fig. 3.2). VP2 polypeptides of LT- and AC-IPNV appear distinctly different from that of Ja, C1, C2, BC1 and VR 299 IPNV isolates. In LT- and AC-IPNV, the VP2 consists of 2 unequal size polypeptides named as VP2(a) and VP2(b) which appeared clearly and consistently in SDS-PAGE. However,LT differs from AC-IPNV with respect to size of the VP2 polypeptides with LT-IPNV VP2(a) having mw of 51K and VP2(b) 49K while those of AC-IPNV being larger with 53K and 51K. The VP2(a) was larger in both isolates constituting 72 to 73% of the total VP2. However VP3 polypeptide of LT-IPNV was comparable in mw (30K) with that of AC-IPNV.

Densitometry data (Table.3.1) also indicated that VP2 of LT and AC-IPNV isolates consists of two unequal sized polypeptides. Approximate percentage contribution of VP2(a) and VP2(b) to VP2 were 44.32 and 16.67 respectively in both LT and AC-IPNV. Together VP2(a) and VP2(b) make up 61% of the total virion polypeptides.

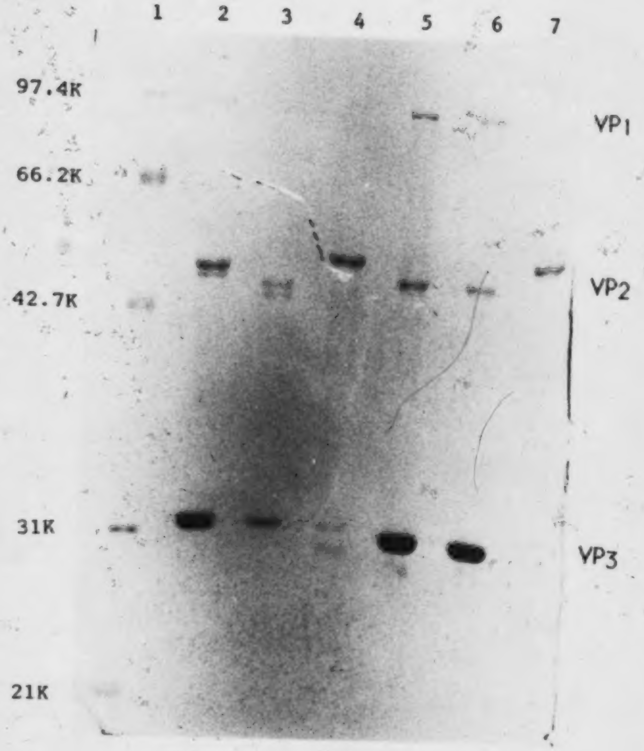


Fig. 3.2. Analysis of IPNV proteins by SDS-PAGE. IPNV isolates purified in CsCl were treated with sample buffer containing 5% 2-mercaptoethanol and 2% sodium dodecyl sulfate at 95°C for 3 min and electrophoresed in 10% polyacrylamide gel at 100 volts for 6 h in 14.4 mM Tris glycine buffer, pH 8.8. The gel was stained with Coomassie blue. Lane 1.standard protein markers; 2. AC-IPNV; 3. LT-IPNV; 4. Ja-IPNV; 5. C1-IPNV; 6. C2-IPNV; 7. BC1-IPNV.

	Polypeptide percentage				
Virus	VP1	VP2			VP3
		a	b	a+b	
LT IPNV	3.08	44.32	16.67	60.99	35.91
AC IPNV	3.30	44.56	16.37	60.93	35.76

Table 3.1. Analysis of LT and AC IPNV polypeptides by densitometer. CsCl purified virus isolates were treated with sample buffer containing 2% SDS and 5% 2-mercaptoethanol and electrophoresed in 10% SDS polyacrylamide gel for 6 h at 100 volts. After thorough destaining protein bands were scanned in a Chromoscan-3 densitometer. Percentage composition of protein was estimated from the corresponding integral area of peaks on the scan.

Cross-neutralization of IPNV isolates with rabbit antisera

Table 3.2 shows cross-neutralization titre of LT, AC and Ja-IPNV isolates by homologous and heterologous antisera. Neutralization (log ND_{50}) by LT-IPNV antiserum of LT- and AC-IPNV were 4.68 and 4.65 respectively. Compared to LT- and AC-IPNV, a lower dilution of LT-IPNV antiserum was required to neutralize Ja IPNV (ND_{50} 3.0). Similarly, AC-IPNV antiserum neutralized LT and AC-IPNV isolates at higher dilutions compared to the Ja-IPNV isolate. Log ND_{50} of Ja-IPNV antiserum with homologous virus was 4.2 whereas the values for LT- and AC-IPNV were 0.86 and 2.96 respectively. Antigenic relationships (1/r) determined using cross-neutralization ND_{50} titers indicated that LT-IPNV is similar but not identical to AC-IPNV (1/r=3.43). The 1/r value between LT and Ja-IPNV is 126 indicating significant differences between them. The overall results indicate that, Ja isolate is distinctly different from LT and AC and that there exist minor antigenic differences between AC- and LT-IPNV isolates.

Production of hybridomas producing antibody to LT-IPNV

A total of 2300 hybridomas were obtained from eight fusions of which 300 were producing antibody specific to LT-IPNV as tested in ELISA. After minicloning thrice, clones that were producing IgM antibodies and also those making antibodies to CHSE-214 cell antigen were screened out. Finally 28 stable clones were selected for analysis of epitope specificity.

Epitope specificity of LT-IPNV MAbs

Epitope specificity of MAbs was determined by additivity index (AI) calculated from reciprocal binding values of MAbs in ELISA. Binding of MAbs was carried out at antigen saturation point. Antigen saturation points with antibodies

Antiserum	IPNV LT	 AC	 Ja
LT	4.68 (1)	4.65 (3.43)	3.00 (126)
AC	4.20	4.65	2.51
Ja	0.86	2.96	4.20

Table 3.2. Cross neutralization of IPNV isolates by rabbit antisera. Aliquots from serial doubling dilutions of antisera were mixed with equal volumes of $10^{2.2}$ $TCID_{50}$/0.1 ml of virus for 1 h at room temperature. The mixture (0.1 ml) was inoculated to CHSE-214 cell monolayer in 96 well microtiter plates. CPE was observed for 14 d and the neutralizing antibody titer (ND_{50}/O.1 ml) caculated according to Karber (1931). Numbers in parenthesis are 1/r values between the viruses (Archetti and Horsfal, 1950).

were obtained from the saturation curves (Table 3.3). A sample of typical saturation curves obtained with 4 MAbs is shown in Fig. 3.3. Ascites were used at concentrations higher than their antigen saturation points. Reciprocal AI values were calculated as described in the methods, for all the pairs of MAbs (Table 3.4). AI between homologous MAb pairs ranged from less than 0 to 11.90. In order to find a common AI,at which there is no additivity statustically, ANOVA test for 2 way classification was applied. The test was carried out at 5, 10, 15,20, or 25 as common AI for homologous MAb pairs. There was no additivity upto AI 25 with the distribution of AI from 0 to 150. At 95% confidence interval it was found that when AI is 25 each observed data was subjected to vary zero plus or minus 23.97. Therefore, reciprocal AI values more than 25 was considered as Mabs reacting with different epitopes on the virus. A LT-IPNV non-specific ascitic fluid used as a control neither gave a background nor interfered with the binding of the specific antibodies. In all, 15 MAbs which had reciprocal AI values >25% were chosen as having specific reactions with different epitopes on the virus.

Polypeptide specificity of LT-IPNV MAbs from the 15 hybridomas

Fig. 3.4 lane A shows the three polypeptides of LT-IPNV- VP1 (mw 96K), VP2 (mw 51K), and VP3 (mw 30K). In the Western blot all the 15 MAbs reacted only with VP2 polypeptides of the virus. Both the bands of VP2 were reactive with the MAbs. The virus polypeptide was solubilized and reduced before reacting with MAbs in Westernblot. The 15 MAbs were named from LT1 to LT15, the isotype and neutralization properties of which are shown in Table 3.5. MAbs LT5, LT6, LT8 and LT11 reacted weakly with VP2 polypeptides as indicated by pale purple band which persisted for only a few hours. LT1 and LT12 reacted moderately with VP2 polypeptide. MAbs LT2, LT3 ,LT4, LT7, LT9, LT10, LT13, LT14 and LT15

MAbs	Saturation point (Reciprocal of dilution of ascites)	Reciprocal dilution used in ELISA
A1P1-75	320	200
A1P1-45	20	10
A1P1-46	80	50
A1P2-39	320	300
A1P2-76	320	300
A2-25	160	100
A2-34	160	100
A2-75	80	80
A2-10	80	50
A2-18	40	30
A2-86	80	50
A2-90	40	30
A2-73	640	600
A3-43	80	50
A3-42	80	50
A2P2C8	80	50
A3-45	160	100
A3-53	160	100
A3-66	80	50
A3-71	80	50
A5-8	160	100
A5-48	80	50
A5-67	80	50
A6-56	80	50
A6-57	40	40
A1P1D1	80	50
A4-18	160	100
A1P2-15	160	100

Table 3.3. Saturation points of LT-IPNV MAb ascites with LT-IPNV antigen obtained from saturation curves. Dilution of ascites on the curve where plateau starts were taken as saturation points.

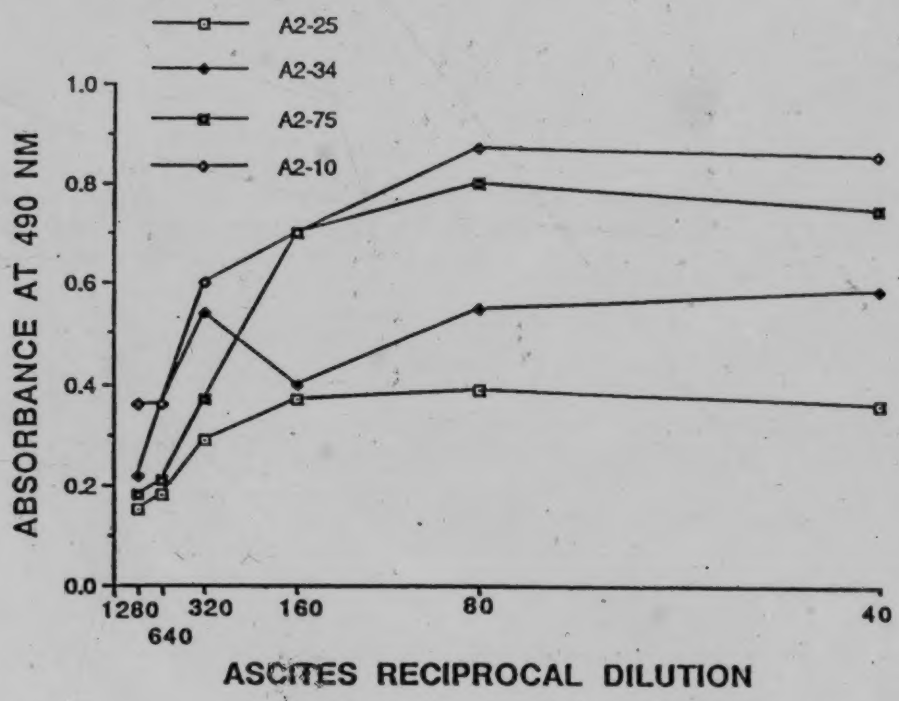


Fig. 3.3. Antigen saturation curves of LT-IPNV MAbs determined by ELISA. Immunolon I plates were coated with 100 μl of $10^{7.5}$ $TCID_{50}$/ml of LT IPNV, and incubated with serial two fold dilution of LT-IPNV MAbs ascites (A2-10, A2-25, A2-34, and A2-75) for 1 h. The plates were washed and incubated with IgG-HRP followed by incubation with substrate. Colour developed in 20 min was measured at 490 nm and antigen saturation curves drawn by plotting absorbance against ascites dilutions.

MAb2 MAb1	LT1	LT2	LT3	LT4	LT5	LT6	LT7	LT8	LT9	LT10	LT11	LT12	LT13	LT14	LT15	LT0
LT1	NA	89.20	36.19	82.20	39.00	33.40	46.28	36.99	39.24	73.29	49.20	36.73	43.29	40.40	83.96	102.00
LT2	34.72	9.25	45.54	35.68	35.52	63.00	56.06	66.00	83.93	43.97	37.24	79.20	83.74	24.46	39.47	102.00
LT3	83.37	84.26	7.95	45.87	58.97	33.39	60.90	121.85	101.97	33.20	79.68	140.76	165.31	86.60	30.23	79.00
LT4	51.36	40.42	74.85	9.88	30.68	79.67	42.59	55.56	107.60	46.76	31.70	42.61	115.70	67.05	66.51	93.00
LT5	39.28	62.02	63.39	88.31	NA	33.42	67.29	48.80	53.00	78.20	76.67	45.06	119.50	44.21	86.20	78.00
LT6	37.62	32.22	66.60	30.40	31.50	11.90	39.15	37.19	87.80	36.90	31.90	56.96	88.46	34.93	55.00	98.00
LT7	42.51	58.40	56.39	43.68	70.96	80.60	9.88	45.86	105.00	65.41	66.02	90.00	156.49	77.09	61.95	90.00
LT8	43.97	56.14	97.47	50.38	84.54	58.44	149.00	10.50	32.00	112.00	86.76	36.03	152.00	46.13	51.60	102.00
LT9	72.14	38.90	36.40	29.67	48.00	33.70	34.20	29.04	NA	40.70	29.22	25.77	49.09	33.00	56.15	89.00
LT10	39.12	42.43	30.94	41.30	63.61	44.42	35.00	33.92	38.19	7.11	31.21	41.00	108.00	46.15	73.86	85.00
LT11	98.00	43.44	76.18	32.14	33.20	70.40	30.71	97.05	59.71	32.17	8.42	86.92	137.50	67.88	30.20	104.00
LT12	63.17	34.98	49.20	59.20	30.68	70.65	44.56	63.20	78.25	83.17	95.60	NA	61.86	53.83	48.40	98.00
LT13	39.00	49.20	68.20	43.98	71.20	66.47	74.89	56.62	47.26	73.00	46.39	81.36	11.00	81.25	81.20	113.00
LT14	36.07	83.28	50.22	63.46	91.28	32.89	39.28	46.29	42.22	48.20	77.78	54.89	47.11	NA	77.24	93.00
LT15	48.66	24.98	66.28	37.20	33.25	36.53	42.16	59.34	83.20	73.28	66.29	52.33	53.39	73.92	NA	112.00
LT0	93.00	88.00	109.00	87.00	87.00	93.00	85.00	94.00	78.00	107.00	107.00	105.00	78.00	112.00	115.00	NA

Table 3.4. Additivity index (AI) of LT-IPNV MAbs produced by 15 different clones. AI values were derived from reciprocal blocking ELISA according to Friguet *et al.* (1983) where, AI= 100 x ([(2 x A of MAb_1 and MAb_2 together)/(A of MAb_1) + (A of MAb_2)] - 1). MAb_1-MAb applied first; MAb_2-MAb applied second; NA-No additivity

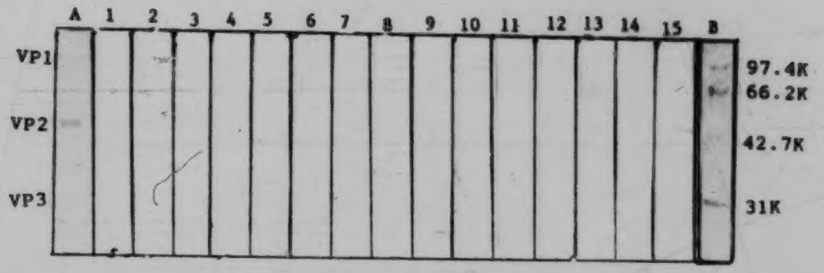


Fig. 3.4. Polypeptide specificity of LT-IPNV MAbs in Western blot. CsCl purified LT-IPNV was treated with sample buffer containing 5% 2-mercaptoethanol and 2% SDS, and electrophoresed in 10% polyacrylamide gel at 100 volts for 90 min. Proteins were transfered to nitrocellulose paper and the paper cut into strips. Strips were incubated with hybridoma culture supernatant followed by incubation with rabbit antimouse IgG-HRP and substrate. Lanes A and B LT-IPNV proteins and standard protein markers respectively stained with amido black ; Lanes 1 to 15 - reaction of LT-IPNV MAbs with VP2 polypeptide.

MAbs	Isotype	Neutralization of LT-IPNV
LT1	G2a	-
LT2	G1	-
LT3	G2a	+
LT4	G3	+
LT5	G1	+
LT6	G1	+
LT7	G3	+
LT8	G3	+
LT9	G3	-
LT10	G1	+
LT11	G1	+
LT12	G2b	+
LT13	G2a	+
LT14	G1	-
LT15	G1	-

Table 3.5. Isotype and neutralization properties of LT-IPNV MAbs. Hybridoma producing specific antibody to LT-IPNV were minicloned thrice and antibody from the stable clones isotyped using Hyclone Laboratory kit (Logan, Utah, USA). Neutralization assay was carried out by mixing equal quantities of the hybridoma culture supernatant and $10^{2.2}$ $TCID_{50}$/0.1 ml of LT-IPNV. The mixture (0.1 ml) was inoculated to CHSE-214 cells monolayer in 96 well microtitre plates. Culture supernatant in which there was no CPE for 14 d was considered to be neutralization positive.

reacted strongly as evidenced by a dark purple VP2 band.

Immunodot reaction of LT-IPNV MAbs with Canadian IPNV isolates

The assay was standardized with optimal concentration of reagents and reaction time with LT-IPNV. The reaction pattern in a typical assay is shown in Fig. 3.5 where isolates could be distinguished by the five Mabs. Results of reaction of 15 LT-IPNV MAbs with various Canadian isolates is shown in Table 3.7.

LT5 and LT13 reacted specifically with LT-IPNV, whereas LT4 reacted with both LT and AC-IPNV. LT3, LT6, LT7, LT8, LT10, LT11 and LT12 reacted with only LT, AC and C2 IPNV isolates. LT2 reacted with all isolates except VR 299 and Ja IPNV isolates, whereas LT9 and LT14 reacted with all isolates except Ja-IPNV. LT15 reacted with all isolates except C1, while LT1 reacted with all isolates uniformly. Using LT5 and LT13 it was possible to differentiate LT-IPNV from AC-IPNV. Using the panel of 15 LT-IPNV MAbs, LT IPNV could be distinguished from other IPNV isolates from their reaction pattern. In addition, LT-IPNV MAbs could differentiate all of the Canadian isolates, particularly C2 and C3. Similar result was obtained with 100 x concentrated antigen of AC, C1, C2, VR 299, Ja, and BC1-IPNV.

Neutralization of IPNV isolates by LT-IPNV MAbs

Of the 15 LT-IPNV MAbs, 10 were neutralizing LT-IPNV (Table 3.8). LT5, LT7 and LT13 neutralized only LT-IPNV and did not neutralize AC-IPNV, or other isolates used in the assay. However, of the ten, seven MAbs- LT3, LT4 , LT6, LT8 LT10, LT11 and LT12 neutralized both LT and AC-IPNV. LT3 and LT12 weakly neutralized AC compared to LT-IPNV. Among the above seven, LT3 and LT4 specifically neutralized LT and AC-IPNV isolates but not C2, which was

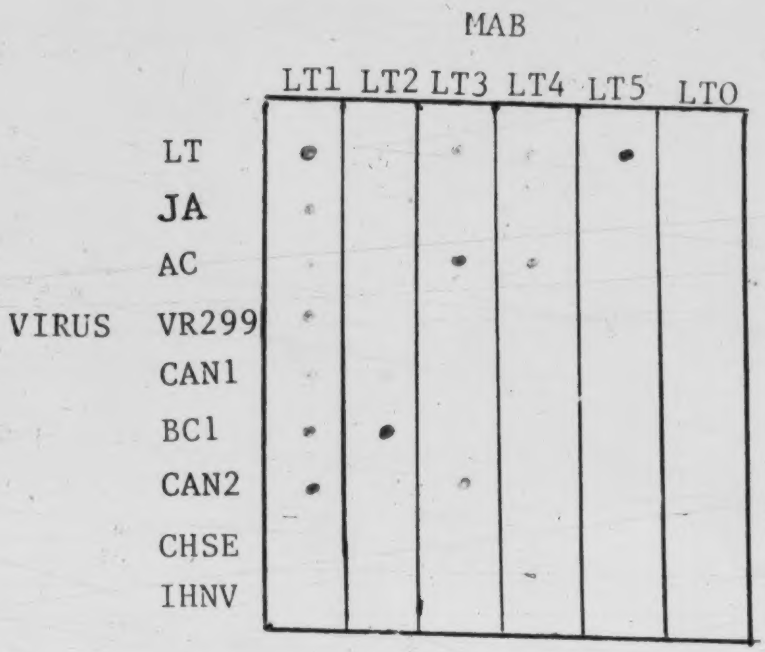

MAB
LT1
LT2
LT3
LT4
LT5
LTO
VIRUS
LT
JA
AC
VR299
CAN1
BC1
CAN2
CHSE
IHNV

Fig. 3.5. Immunodot assay of IPNV isolates with LT-IPNV MAbs. Dots consisting of 2 μl of virus culture supernatant were made on nitrocellulose paper and the paper incubated with LT-IPNV hybridoma culture supernatant followed by incubation with rabbit antimouse IgG-HRP and substrate. Colour developed in 5 min was photographed.

	MAbs														
virus	LT1	LT2	LT3	LT4	LT5	LT6	LT7	LT8	LT9	LT10	LT11	LT12	LT13	LT14	LT15
LT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AC	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+
C2	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+
VR299	+	-	-	-	-	-	-	-	+	-	-	-	-	+	+
Ja	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
BC1	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+
C1	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-

Table 3.7. Immunodot assay of IPNV isolates using LT-IPNV MAbs. IPNV isolates were grown in CHSE-214 cells and 2 μl culture supernatant (10^8 $TCID_{50}$/ml) dotted on to nitrocellulose paper. The paper was incubated with hybridoma culture supernatant followed by incubation with IgG-HRP and substrate. Purple colour developed in 5 min was recorded.

	MAbs									
Virus	LT3	LT4	LT5	LT6	LT7	LT8	LT10	LT11	LT12	LT13
LT	++	+++	+++	+++	+++	+	++	++	+++	+++
AC	+	+++	-	+++	-	+	++	+++	++	-
C2	-	-	-	+++	-	+	+	+++	+++	-
C1	-	-	-	-	-	-	-	-	-	-
Ja	-	-	-	-	-	-	-	-	-	-
BC1	-	-	-	-	-	-	-	-	-	-
VR299	-	-	-	-	-	-	-	-	-	-

Table 3.8. Neutralization of IPNV isoates by LT-IPNV MAbs. Equal quantities of $10^{2.2}$ TCID$_{50}$/0.1 ml of LT-IPNV and serial two fold dilutions of hybridoma culture supernatant were mixed and 0.1 ml inoculated to CHSE-214 cell monolayers in 96 well microtiter plates. CPE was observed for 14 d and ND$_{50}$ calculated according to the method of Karber (1931). ND$_{50}$>3, + + + ; ND$_{50}$>2, + + ; ND$_{50}$>1, + ; No detectable neutralization, -.

neutralized by 5 MAbs-LT6, LT8, LT10, LT11, and LT12. The other four IPNV isolates, Ja, BC, C1 and VR 299 were not neutralized by any of the LT-IPNV neutralizing MAbs. Thus these neutralizing MAbs could clearly distinguish LT-IPNV from AC-IPNV and also C3 from C2 IPNV.

Table 3.9 gives the pooled results of immunodot and neutralization assay. The results of immunodot assay, in general agrees with that of neutralization assay. However, LT7 which reacted positively with LT, AC and C2 IPNV isolates in immunodot assay, neutralized only LT, not the other two. In both the assays, LT5 and LT13 reacted specifically with LT-IPNV, whereas LT4 reacted with both LT and AC-IPNV.

Immunodot reaction of LT-IPNV MAbs with IPNV isolates from Alberta and Manitoba

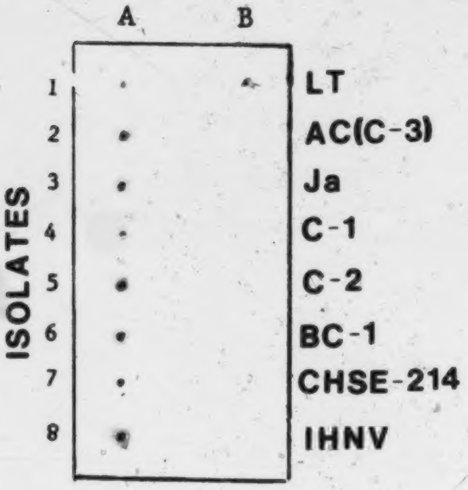
Eight IPNV isolates from Alberta and two from Manitoba were tested with LT5 and LT13-MAbs specific to LT-IPNV. Fig. 3.6 shows immunodot reaction of LT5 MAb with IPNV isolates from Alberta. All the isolates from Alberta and Manitoba reacted positively in immunodot assay indicating that these isolates possess two specific epitopes similar to that of LT-IPNV from Cornwall Lake. All the six isolates from lake trouts of Cornwall Lake also reacted with LT5 and LT13 MAbs. In the control, as observed before only LT-IPNV reacted positively with LT5 and LT13 while Ja, AC, C1, C2, BC1 did not react.

3.4 DISCUSSION AND CONCLUSION

IPNV possess three major polypeptides, VP1 (105K), VP2 (54K) and VP3 (31K) contributing 4%, 62% and 34% respectively to the total viral protein (Macdonald and Dobos, 1981; Dobos and Roberts, 1982). LT-IPNV also has been

	LT-IPNV MAbs														
Virus	LT1	LT2	LT3	LT4	LT5	LT6	LT7	LT8	LT9	LT10	LT11	LT12	LT13	LT14	LT15
LT (a)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(b)	-	-	++	+++	+++	+++	+++	+	-	++	++	+++	+++	-	-
AC (a)	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+
(b)	-	-	+	+++	-	+++	-	+	-	++	+++	++	-	-	-
C2 (a)	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+
(b)	-	-	-	-	-	+++	-	+	-	+	+++	+++	-	-	-
VR299 (a)	+	-	-	-	-	-	-	-	+	-	-	-	-	+	+
(b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ja (a)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
(b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BC1 (a)	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+
(b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C1 (a)	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-
(b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.9. Comparison of immunodot reaction and neutralization capacity of LT-IPNV MAbs with IPNV isolates. (a) Immunodot assay, + positive reaction; - no reaction. (b) Neutralization, + + + $ND_{50} > 3$; + + $ND_{50} > 2$; + $ND_{50} > 1$;- no detectable neutralization.



1. Raven Hatchery brood rainbow trout IPNV
2. Charles Lake wild lake trout IPNV
3. Cold Lake Hatchery rainbow trout IPNV
4. Bow River rainbow trout IPNV
5. Sam Livingston Hatchery rainbow trout IPNV
6. Cold Lake Hatchery sentinel brook trout IPNV
7. Cold Lake white fish IPNV
8. Wylie Lake lake trout IPNV

Fig. 3.6. Immunodot assay of IPNV isolates from different regions in Alberta with LT-IPNV specific MAb LT5. IPNV isolates were grown in CHSE-214 cells and the culture supernatants dotted on to nitrocellulose paper. The paper was incubated with MAb LT5 followed by incubation with IgG-HRP and substrate. Colour developed in 5 min was photographed.

shown to consists of 3 polypeptides VP1 (96K), VP2 (51K) and VP3 (30K) contributing 3.08%, 60.99%, and 35.91% respectively to the construction of virion. The VP2 polypeptide showed two bands a and b contributing 44.3% and 16.67% respectively to make up 60.97% of the total virion protein. Since both bands of LT-IPNV VP2 reacted with all the MAbs, it is likely that they have a common origin.

Among the different IPNV isolates tested by SDS-PAGE only VP2 of AC-IPNV showed two bands similar to that of LT-IPNV. However, Macdonald *et al.* (1983) in their studies on AC-IPNV did not observe this double band in the VP2 polypeptide region of the gel. The VP2 of LT and AC-IPNV isolates, however, could be differentiated by differences in moleular weight and appearance of bands. LT-IPNV VP2 has a low mw (51K) compared to that in AC-IPNV (53K). Since the bands occured consistently in all the SDS-PAGE they can be used to differentiate and identify LT and AC-IPNV isolates from rest of IPNV isolates in Canada. The VP3 polypeptides of LT and AC IPNV were similar in mw (30K) to Ja-IPNV but the isolates did not show the characteristic double bands of VP3 of the latter. It appears that LT and AC-IPNV isolates have undergone changes in their VP2 polypeptide-the outer capsid protein and thus may have diverged slightly in evolution.

Three distinct but related serotypes of IPNV : Sp, Ab and VR 299 had been defined previously by using rabbit antisera (Hill, 1976; Macdonald and Gower, 1981; Okamoto *et al.*, 1983). Further analysis of over 175 isolates from wider geographical regions and host range, has indicated that there may be at least 9 serotypes within one serogroup, and one serotype within a second serogroup (Hill and Way, 1983). Later, epitope analysis of North American isolates using monoclonal antibodies directed against West-Buxton IPNV revealed antigenic differences among five isolates from USA and one from Canada which were all earlier classified as VR 299 (Caswell-Reno *et al.*, 1986). Hill and Way (1988) proposed 4 IPNV serotypes in

Canada- C1, C2, C3 and Ja. Subsequently, by using a panel of MAbs in immunodot assay Caswell-Reno et al.(1989) demonstrated the existence of three serotypes C1, C2, and Ja. Their study could not establish C3 as a distinct serotype.

Cross neutralization studies conducted using rabbit antisera indicated that LT-IPNV is similar and not identical to AC-IPNV but distinctly different from Ja IPNV. Antigenic relationships (1/r) derived from ND_{50} values further support the relationship among the three isolates. The 1/r values between LT and AC -IPNV was 3.4, compared to 126 between LT and Ja-IPNV. The value of 1/r gives the extent of antigenic differences between 2 viruses when both viruses and antisera are used in cross neutralization tests. Kelly and Neilsen (1990) in their cross-neutralization studies using rainbow trout antisera observed that LT and AC-IPNV are identical, which is probably due to cross reaction of IgM like antibody in rainbow trout antisera.

In order to resolve antigenic differences between the closely related LT and AC-IPNV, monoclonal antibodies were made to the former for epitope analysis. Fifteen MAbs were selected by virtue of their higher reciprocal AI values in ELISA which indicates their specificity to different epitopes (neutralizing and non-neutralizing) on VP2 polypeptide. Additivity index of each antibody combination compares the amount of two MAbs binding together to virus together with the sum of the amount of each MAb binding separately (Friguet *et al.*, 1983). Theoretically, AI for 2 MAbs that bind independently at different sites should be equal to 100, whereas 2 MAbs that bind at the same site and or completely block each other should yield an AI of zero. AI values between 0 and 100 therefore reflect relative degrees to which one MAb interferes with the binding of the other MAb. AI values more than 25 was considered to select MAbs pairs as in some cases reciprocal AI values up to 10 was obtained with the same MAb. AI values of MAb pairs ranged from less than 0 to more than 100% indicating inhibition or augmentation of binding of MAbs. This may be due to steric effect where binding of one antibody influences the subsequent binding of another (Friguet *et al.*, 1983; Lussenhop *et al.*, 1988) or attachment of one antibody causes allosteric alteration of the adjacent antigenic deteriminant (Lubeck and Gerhard, 1981). AI values less than 100 were noticed due to interference in binding between MAbs which indicate overlapping epitopes or close proximity between them. The AI values of LT0 with other MAbs was high indicating the validity of the values obtained in the experiment.

All fifteen MAbs reacted with VP2 polypeptide of LT-IPNV in the Westernblot. The virus protein was solubilized in 2% SDS and reduced with 5% mercaptoethonol. Nine MAbs (LT2, LT3, LT4, LT7, LT9, LT10, LT13, LT14 and LT15) reacted strongly in the Westernblot. On the other hand LT1 and LT12 reacted moderately and LT5, LT6, LT8 and LT11 weakly in the Westernblot. This reaction pattern in Westernblot suggests the presence of both conformation and sequence dependent epitopes on LT-IPNV VP2 polypeptide similar to epitopes on West Buxton IPNV (Caswell-Reno *et al.*, 1986), Sp IPNV (Wolski *et al.*, 1986), N1 IPNV serotype from Norway (Christie *et al.*, 1990) and IBDV strains from Australia and U.K. (Azad *et al.*, 1987; Becht *et al.*, 1988).

Out of 15 LT-IPNV MAbs 10 neutralized LT-IPNV. It was noticed that 50% of MAbs which reacted strongly in Westernblot did not neutralize the virus (Table 3. 6). On the other hand, 50% of MAbs which reacted very weakly in the Westernblot showed very good neutralizing activities. Hybridoma culture supernatant having uniform antibody titer as determined by ELISA was used in both the Western blot and neutralization assays. These results indicate that MAbs that bind strongly in the Western blot do not have good neutralizing activity or vice versa.

MAbs	Westernblot reaction	Neutralization of LT-IPNV
LT1	++	-
LT2	+++	-
LT3	+++	++
LT4	+++	+++
LT5	+	+++
LT6	+	+++
LT7	+++	+++
LT8	+	+
LT9	+++	-
LT10	+++	++
LT11	+	++
LT12	++	+++
LT13	+++	+++
LT14	+++	-
LT15	+++	-

Table 3.6. Comparison of Westernblot reaction and neutralization capacity of LT-IPNV MAbs. Westernblot reaction: pale purple +, purple + +, dark purple + + +. Neutralization test: $ND_{50}>3$ + + +, $ND_{50}>2$ + +, $ND_{50}>1$ +, no neutralization -.

The panel of 15 LT-IPNV MAbs reacted differently with various IPNV isolates in immunodot assay. LT5 and LT13 reacted specifically with LT-IPNV indicating that the virus has its own unique epitopes. LT4 reacted with both LT and AC IPNV, by recognizing a common epitope shared by these two isolates. Seven MAbs LT3, LT6, LT7, LT8, LT10, LT11, LT12 could recognize common epitopes on LT, AC and C2, which indicate that these three isolates are antigenically closely related. In the immunodot reaction, LT5 and LT13 clearly distinguished LT and AC IPNV isolates and LT4 between C2 and C3 (AC-IPNV). Caswell-Reno *et al.* (1989) using a panel of MAbs in the immunodot reaction showed epitope differences between the proposed Canadian IPNV serotypes, C1, C2, and Ja but not between C2 and C3. Reaction of the six IPNV isolates from Cornwall Lake with LT-IPNV specific MAbs-LT5 and LT13 indicated that the isolates in the lake are identical to LT-IPNV with respect to these two epitopes. In general, using the panel of 15 MAbs in immunodot reaction, LT-IPNV could be clearly distinguished from AC, and other Canadian isolates.

A panel of 10 MAbs LT3, LT4, LT5, LT6, LT7, LT8, LT10, LT11, LT12 and LT13 neutralized LT-IPNV. Among them LT5, LT13 and LT7 were specific only to LT-IPNV. LT7 reacted positively by binding to LT, AC and C2 in the immunodot test, but could neutralize only LT-IPNV in the neutralization assay. Caswell-Reno *et al.* (1986) observed a similar phenomenon, where in Reno and Buhl IPNV isolates reacted with WB-IPNV MAbs in ELISA, but were not neutralized. A similar phenomenon was also observed with poliovirus (Emini *et al.*, 1983) where MAb prepared against type 1 Sabin strain was able to bind to both Mahoney and Sabin strain but was capable of neutralizing only Sabin strain. Seven neutralizing MAbs LT3, LT4, LT6, LT8, LT10, LT11 and LT12 neutralized both LT and AC, indicating presence of large numbers of common epitopes on the isolates.

LT3 and LT4 neutralized LT and AC-IPNV but not C2, which is similar to the immunodot reaction. Five MAbs, LT6, LT8, LT10, LT11 and LT12 neutralized LT, AC and C2 IPNV isolates. The reaction pattern of these three isolates (LT, AC, and C2) with LT-IPNV MAbs, was similar in the immunodot and the neutralization assays.

MAbs that neutralized LT-IPNV did not neutralize Ja, BC1, C1 and VR 299 isolates. It should also be noted that these LT-IPNV neutralizing antibodies did not bind to the above viruses in the immunodot test.

Recently, several IPN outbreaks have been recorded in trout hatcheries in Alberta and it is believed that IPNV from Cornwall Lake is the possible source of the virus. An immunodot assay was performed to identify eight IPNV isolates collected from different hatcheries and lakes in Alberta using LT-IPNV specific MAbs LT5 and LT13. A positive reaction with all the eight isolates indicates that the isolates are identical to LT-IPNV with respect to the two epitopes. Therefore, these IPNV isolates may have originated from lake trout from Cornwall Lake. Lake trout from Cornwall Lake was used as a source of reproductive products from 1982 to 1987. Lake trout eggs collected were hatched in Cold Lake Hatchery and fingerlings had been distributed to other hatcheries and lakes in Alberta.

Two IPNV isolates from Manitoba- Grand Rapids and HM also reacted with LT-IPNV specific MAbs and hence they are identical to LT-IPNV from Cornwall Lake.

In summary, LT-IPNV has characteristically two polypeptides in the VP2 region which is found to be similar only to that of AC IPNV. However, VP2 polypeptides of LT-IPNV differ from that of AC-IPNV by possessing lower molecular weights. The VP2 polypeptides of these isolates can be used to differentiate them by SDS-PAGE. Cross neutralization assay using rabbit antisera indicated that LT and AC-IPNV isolates are nearly similar to each other, but differ distinctly from Ja IPNV. However, LT-IPNV monoclonal antibodies clearly distinguished LT IPNV from AC-IPNV and also from other Canadian isolates. In a panel of 15 MAbs recognizing different epitopes on VP2 polypeptide of LT-IPNV, two MAbs LT5 and LT13, specifically reacted with LT-IPNV in the imunodot assay. In the neutralization assay three MAbs, LT5, LT13 and LT7 were found to be specific to LT-IPNV. In addition, the proposed C2 and C3 could be clearly distinguished using MAbs LT3 and LT4. The LT-IPNV specific MAbs were used to confirm the spread of the virus from Cornwall Lake to other regions in Alberta.

From the above results it can be concluded that LT-IPNV is antigenically distinct from AC, Ja and other Canadian IPNV isolates and may have been originated indigenously in Cornwall Lake.

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CHAPTER 4

Characterization of LT-IPNV gene encoding VP2 polypeptide

4.1 INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) causes a serious disease in young salmonids reared under hatchery conditions. The virus is icosahedral, non-enveloped and possesses a double-stranded RNA genome which consists of two segments, A and B. Segment A encodes a capsid protein, VP2, a non-structural protein, NS, and an internal protein, VP3, in a single large open reading frame (ORF), and segment B encodes VP1, a putative RNA polymerase (Duncan *et al.*, 1987; Nagy *et al.*, 1987). The order of regions coding for the three proteins by the segment A large ORF is 5'-PVP2-NS-VP3-3'as determined in IPNV from Jasper (Duncan and Dobos, 1986). Though the precise coding boundaries are not known, a 1680 bp region in segment A is believed to encode the VP2 polypeptide.

LT-IPNV was first isolated in 1984 from Cornwall Lake, Alberta. The isolate was of interest with respect to its antigenic and molecular relationships with other IPN virus isolates in Canada. Studies conducted in our laboratory showed that 44.4% of adult lake trout in Cornwall Lake were infected with the virus. The virus is pathogenic to the young of other salmonids, but interestingly, not to its natural host. The VP2 polypeptide of the virus is similar to that of IPNV from Arctic char (AC-IPNV) in consisting of two unequal bands, but differs from it by posessing two slightly lower apparent molecular weights. In cross neutralization with rabbit antisera the virus could be differentiated from Ja but not from AC-IPNV. However, by epitope analysis of the VP2 polypeptide using LT-IPNV monoclonal antibodies, LT and AC IPNV isolates could be clearly differentiated by two binding and three neutralizing epitopes. This chapter reports further characterization of LT-IPNV by sequence analysis of two regions in the VP2 gene and its comparison with those of AC and Ja IPNV isolates.

4.2 MATERIALS AND METHODS

Purification of virus

LT, AC and Ja IPNV isolates were plaque purified thrice and propagated in CHSE-214 cells at a low multiplicity of infection. The virus isolates were purified by centrifugation in CsCl gradients according to the method of Chang *et al.* (1978), as described in chapter 3.

RNA extraction

Virus RNA was extracted according to Macdonald and Yamamoto (1977). Virus purified by isopycnic sedimentation in CsCl was dialysed in TNE buffer (pH 7.3) and 250 μl of it was treated with 50 μl of proteinase K (10 mg/ml) and 25 μl of 10% SDS at 50^{O}C for 90 min to digest the virus protein. RNA from the above mixture was extracted thrice with liquified phenol, and the supernatant separated after centrifuging was precipitated with 0.1 vol of 3 M sodium acetate and 3 vol of ethanol at -20^{O}C overnight. The precipitated RNA was pelleted, redissolved in distilled water, and its concentration determined by measuring its absorbance at 260 nm. An absorbance of 1 at 260 nm was considered to be equal to 50 μg of RNA.

Equal quantities of RNA of the three isolates were electrophoresed at 5 volts/cm for 4 h in a 1.25% agarose gel. The gel was stained with ethidium bromide and the RNA bands visualised under UV light were photographed using Polaroid type 667 film.

Selection of primers for polymerase chain reaction (PCR)

RNA sequences from segment A of Ja IPNV (Duncan and Dobos, 1986) and infectious bursal disease virus of chickens (IBDV) (Azad *et al.*, 1985) were compared to find homologous regions using the PC gene program. Primers of 23 to 24 nucleotides were selected from the conserved regions within the VP2 encoding region of Ja-IPNV and synthesized on a 381A DNA synthesizer (Applied Biosystems, San Fransisco). At first primers 1 and 2, with an interprimer spacing of 800 bp, were used in PCR. After failure to obtain a PCR product in several attempts, a third primer was chosen and used with primer two. Primers 4 and 5 were chosen from a region in VP2 which was shown to possess a highly antigenic site by hydrophilicity mapping according to Hopp and Woods with the PC gene program. Table 4.1 gives particulars of the primers used for PCR.

Preparation of cDNA

cDNA was synthesized according to the method of Cashdollar *et al.* (1982). Approximately 2-3 μg of purified RNA was dissolved in 2.5 μl of 10 mM Tris-HCl, pH 7.8 and mixed with 22.5 μl of dimethyl sulfoxide (DMSO) to a final concentration of 90%. The mixture was heated at 60°C for 15 min to denature the ds RNA and 12.5 μl of it was transfered to 190 μl of reverse transcription mixture, where the DMSO concentration was reduced to 5.6%. The reverse transcription mixture consisted of 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$,70 mM KCl, 30 mM 2-mercaptoethanol, 0.5 mM each of dNTPS, 15-20 units of avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim) at 7.5 units/μg RNA and 200 picomoles each of primers. Reverse transcription was carried out at 42°C for 60 min and the cDNA extracted with 100 μl each of chloroform and phenol. The cDNA product was precipitated with 3 vol of ethanol and 0.1 vol of 3 M sodium

Primer No.	Sequence	No.of bases	Region on VP2 gene	Inter primer space(b)	G+C content(%)
1	5'> TAGGTGTGGGTTTGGGATCAGCTC <3'	24	1260-1236	800	56.52
2	5'> GCTGGTCTGTATGCACTCAATGG <3'	23	453-476	198	54
3	5'> CATCCTTGCTCCGTTCATGGACTG <3'	24	698-674		54
4	5'> CCCAGTCAATCCTGACCGTGGCT <3'	23	1197-1220	282	60.86
5	5'> GGAGCCGCCATTGGGAAGAGCGT <3'	23	1526-1503		65.20

Table 4.1. Details of primers used for polymerase chain amplification of IPNV VP2

acetate at -20°C overnight. After precipitation, DNA was pelleted and treated with 50 μl of 0.5 M KOH for 2 h at 22°C to digest any RNA associated with it. The resulting cDNA was precipitated in ethanol as before and the pellet obtained by centrifugation was dissolved in 100 μl of $dddH_20$.

Polymerase chain amplification of IPNV cDNA

The polymerase chain reaction (PCR) was carried out according to Saiki *et al.* (1988). About 7-10 μl of cDNA, prepared as above, was mixed with 200 picomoles each of primers, 1 mM each of dNTPS, 2 units of Taq DNA polymerase (Boehringer Mannheim) and 10 μl of 10X PCR buffer (700 mM Tris HCl, pH 8.8, 20 mM $MgCl_2$, 1% Triton X-100). The final volume of the reaction mixture was made up to 100 μl with $dddH_2O$. After thorough mixing, 50 μl of mineral oil was layered on the mixture to avoid evaporation and PCR carried out in a Techne PHC-2 machine (Mandel Scientific Co Ltd, Edmonton) as follows. The mixture was heated at 94°C for 3 min to denature the cDNA, followed by 25 cycles, each involving a denaturation step at 94°C for 30 sec, an annealing step at 56-60°C for 1 min and an extension step at 72°C for 2 min. PCR with primers 2 and 3 was carried out at three different annealing temperatures, 56°C, 58°C and 60°C. With primers 4 and 5 PCR was carried out at annealing temperatures of 60°C, 55°C, 50°C and 45°C. At the end of 25 cycles DNA was extracted with 50 μl of chloroform and precipitated by adding 3 vol of ethanol and 0.1 vol of 3 M sodium acetate,then kept at -20°C overnight. One third of the precipitated PCR product (100 μl) was pelleted, redissolved in TE buffer and electrophoresed on a 1.25% agarose gel at 5 volts/cm for 3-4 h. The gel was stained with ethidium bromide and bands were visualised under UV light. *Alu*I digested pAT 153 DNA and *Cla*I digested λDNA were used as standard markers. A Southern transfer of the PCR products to nylon membrane was carried out and the membrane was hybridized with ^{32}P labelled primers. The result of hybridization was assessed by autoradiography.

In the case of LT-IPNV, with primers 2 and 3, two products, one of 200 bp and another of 600 bp were formed. Therefore, the products were separated and amplified further by PCR before sequencing. Two-thirds of the PCR product of LT-IPNV was loaded on a 1.25% low melting agarose gel and electrophoresed for 4 h at 5 volts/cm. The gel was stained with ethidium bromide and the DNA bands were visualised under UV light. The DNA in the agarose gel was excised and then treated with 5 vol of 20 mM Tris-HCl, pH 8.0 and 1 mM EDTA at 65°C for 5 min. The melted agarose was extracted once each with phenol equilibrated with 0.1 M Tris-HCl, pH 8.0, 1:1 phenol and chloroform and chloroform alone. Later, one third of the DNA obtained was amplified further by PCR as described previously.

Sequencing PCR DNA.

a. Direct sequencing of asymmetric PCR DNA

Approximately 2.5 μl of a 100X dilution of PCR product was mixed with 20 picomoles of one primer (as excess primer), 0.2 picomoles of another (as limiting primer), 20 μM each of dNTPs, 1 unit of Taq DNA Polymerase and 10 μl of 10X PCR buffer. The final reaction volume was made up to 100 μl with $dddH_2O$. After thorough mixing 50 μl of mineral oil was layered on the mixture and PCR carried out for 30 cycles as described before for symmetric PCR. The product was precipitated in ethanol and sodium acetate and the pellet obtained by centrifuging was dissolved in 75 μl of $dddH_2O$. The sequencing reactions were carried out with each of the primer pairs, with one of them in excess and the other in limiting concentration.

Asymmetric PCR amplified DNA was sequenced directly by the dideoxy chain termination method of Sanger *et al.* (1977). The DNA in 20 μl of the above preparation was mixed with 4 μl of 10X PCR buffer, 5 μl of ^{32}P labelled limiting primer, 1 unit of Taq DNA Polymerase and 1 μl each of 10% NP 40 and Tween 20. The total volume of the reaction mixture was made up to 34 μl with distilled water and 7.5 μl was dispensed to each of A, G, C, T tubes containing 2.5 μl of ddA, ddG, ddC and ddT termination mixes. The contents of the four tubes were mixed well and the labelled primers annealed at 60°C followed by chain extension at 72°C for 2-3 min. The reaction was stopped by adding 4 μl of sequence loading buffer to each tube.

b. Sequencing of cloned PCR DNA

*Bam*HI digested pUC118 (2 μg) was mixed with 2.5 μl of oligo DSP 9 (5>TATACGCGTG<3), 1.5 μl of oligo DSP 12 (5>GATCCACGCGTATAT<3), 3 μl of 5X ligase buffer, 1 μl of T4 DNA ligase (1 unit/μl) and 5 μl of $dddH_2O$. The mixture was incubated at 11°C overnight and the oligo-ligated vector was precipitated in ethanol, pelleted and redissolved in TE buffer.

Approximately 15 μl of chloroform extracted PCR amplified DNA was mixed with 4 μl of linker modified pUC 118 (0.5 μg), 7 μl of 5X buffer, 1 μl of DNA ligase and 3 μl of $dddH_2O$ then incubated overnight at 10°C. Transformation of *E. coli* with the recombinant plasmid was carried out on ice for 1 h by mixing 35 μl of the above ligated DNA with 150 μl of competent *E.coli* (MV 1193 strain) cells. Later the mixture was added to 1 ml of 2X YT medium followed by incubation for one more hour at 37°C. Transformed cells in 400 μl of mixture were mixed with 50 μl of 2% X-gal and 10 μl of 100 mM IPTG and plated on 2X YT agar containing ampicillin (100 μg/ml) and incubated overnight at 37°C. White colonies were marked and transferred to a nylon membrane layered on 2X YT agar and grown overnight at 37^{o}C. The membrane was treated with 0.5 N NaOH for 7 min, followed by two washes in 1 M Tris HCl (pH 8), once in 0.5 M Tris-HCl (pH 8) plus 1.5 M NaCl for 4 min and once with 2X SSPE for 5 min. The membrane was baked at 80^{o}C for 45 min, followed by a thorough wash in 2X SSPE containing 0.1% SDS for 20 min at 45^{o}C. The membrane was hybridized with ^{32}P labelled primers at 55^{o}C and autoradiographed.

Positive clones were grown in 3 ml of 2X YT medium containing ampicillin (100 μg/ml) overnight at 37^{o}C. A suspension of the culture (1.5 ml) was centrifuged for 30 sec and the pellet obtained was treated with 100 μl of lysozyme solution and 200 μl of lysis solution on ice for 5 min. Later, 150 μl of 3 M potassium acetate was added and the DNA extracted with phenol and 1:1 phenol chloroform, then precipitated with ethanol at -80^{o}C for 1 h. DNA in ethanol was pelleted and redissolved in 100 μl of TE buffer (pH 7.5) and treated with 5 μl of RNase A (2 mg/ml) for 30 min at 37^{o}C. DNA was further extracted once with 1:1 phenol chloroform followed by one extraction with chloroform alone. The DNA was precipitated with 250 μl of ethanol and pelleted by centrifugation. The pellet was redissolved in 100 μl TE buffer and 5 μl of it was electrophoresed on a 1.25% agarose gel and bands visualised by UV light after staining with ethidium bromide.

Cloned plasmid DNA was sequenced with Taq DNA polymerase in a two step extension method (Promega). A 5 μl solution of DNA was diluted with 13 μl of dddH$_2$O and denatured with 1 μl of 2 N NaOH and 2 mM EDTA for 10 min at RT. The denatured DNA was precipitated with 2.5 μl of 3 M sodium acetate and 60 μl of cold ethanol at -20^{o}C for more than 20 min and then pelleted. The dried DNA was dissolved by addition of 5 μl of 5X PCR buffer, 2 μl of either forward or reverse sequencing primers (1 picomole/μl), 2 μl of labelling mix, 5 μl of 5% TMAC, 1 μl of 10% NP 40 and Tween 20, and 8 μl of dddH$_2$O. The mixture was incubated at 48^{o}C for 10 min followed by addition of 1 μl of ^{32}P dATP and 1 μl of Taq DNA polymerase (1 unit/μl) and incubated for 1 min at 48^{o}C. Later, 6 μl aliquots of the above labelled mixture were transferred to four tubes, each containing 1 μl of the respective termination mixes. The contents were mixed well and incubated for 5 min at 70^{o}C then the reactions were stopped by adding 14 μl of sequencing loading buffer to each.

Sequenced DNA from asymmetric PCR or cloned DNA reactions was then denatured at 90^{o}C for 2 min and 2.5 μl samples loaded on to 6% polyacrylamide/8.3 M urea gels. Electrophoresis was carried out in 60 mM TEB buffer at 4000 volts for 3 h and 8 h. The gel was exposed to Kodak XAR X-ray film for appropriate times and developed. The sequence was read and compared with the corresponding nucleotide and amino acid sequence of Ja IPNV using the NALIGN method of Myers and Miller in the PC gene programme.

4.3 RESULTS

Comparison of RNA of IPNV isolates

Fig. 4.1 shows the bisegmented RNA of Ja, LT and AC IPNV isolates. Using ss RNA as markers the apparent molecular size of the RNAs of each isolate was found to be approximately 6 kb. There is no obvious difference in molecular weight among the RNAs of the three isolates in 1.25% agarose gel.

Amplification of DNA by polymerase chain reaction

A PCR product could not be obtained with primers 1 and 2, even after several attempts. However, when primers 2 and 3 were used a product was formed (Fig. 4.2). Interestingly, with Ja and AC IPNV isolates only one product of 200 bp was formed, whereas with LT-IPNV two products, one of 200 bp and another minor

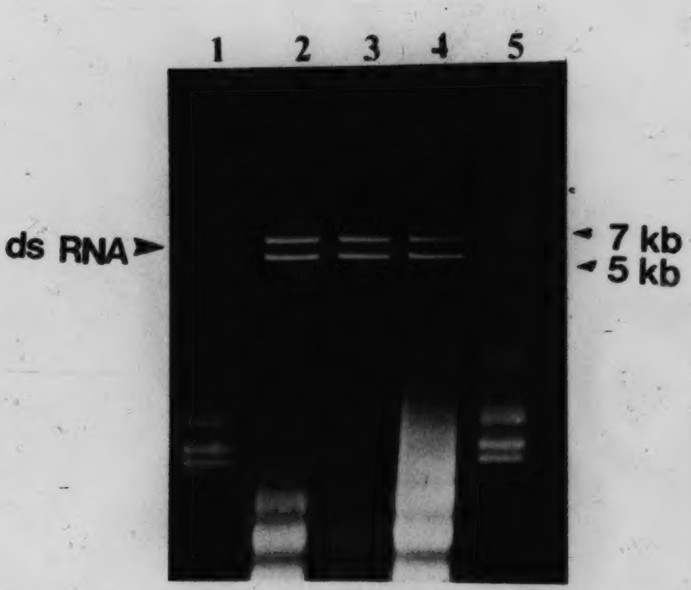

Fig. 4.1. Double stranded RNA of the IPNV isolates. CsCl purified IPNV isolates were treated with proteinase K in the presence of 0.8% SDS, followed by extraction three times with liquified phenol. The RNA extracted was precipitated in ethanol, redissolved and electrophoresed at 5 volts/cm for 4 h in a 1.25% agarose gel. The gel was stained with ethidium bromide and photographed using 667 Polaroid film. Lanes 1 and 5, ss RNA markers ; 2, Ja-IPNV ; 3, LT-IPNV ; 4, AC-IPNV.

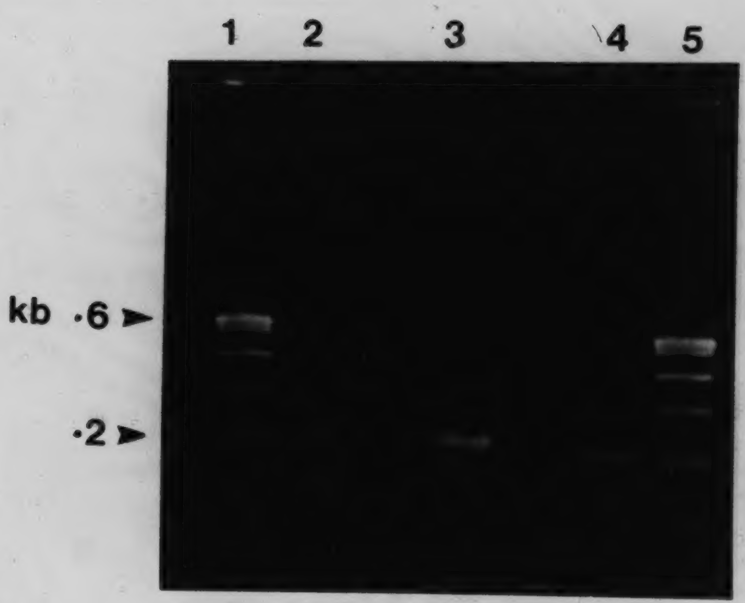

Fig. 4.2. PCR amplification of DNA from nt 476-674 of IPNV-RNA, using primers 2 and 3. cDNA was synthesized using primers 2 and 3 by reverse transcription and amplified by 25 cycles of PCR. Each cycle involved a denaturation step at 94°C for 30 sec, and an annealing step at 58-60°C for 1 min, and an extension step at 72°C for 2 min. One third of the DNA recovered after the chloroform extraction and ethanol precipitation was electrophoresed in a 1.25% agarose gel, and photographed by UV illumination on 667 polaroid film after staining the gel with ethidium bromide. Lanes 1 and 5, DNA markers; 2, Ja-IPNV; 3, LT-IPN; 4, AC-IPNV.

one of 600 bp, were seen. There was no change in the products when the primer annealing temperature was shifted from 56°C to 60°C. The two products with LT-IPNV were separated in 1.25% low melting agarose gel and amplified again by PCR. Results indicate that the separated products generated only the corresponding products when further amplified by PCR (Fig. 4.4). A Southern transfer revealed that the products formed were specific to the primers (Fig. 4.3).

In the case of primers 4 and 5, a PCR product was obtained with Ja IPNV at an annealing temperature of 60°C, but not with either LT or AC IPNV. With the latter two a clear product was obtained only when the annealing temperature was reduced from 60°C to 45°C (Fig. 4.5). There was no DNA synthesis at 60, 55 or 50°C annealing temperatures. Southern hybridization indicated that the products formed are specific to primers 4 and 5 (Fig. 4.6).

Sequence analysis of PCR DNA

Sequencing of PCR products was carried out by using DNA from asymmetric PCR or after cloning. Among the 4 PCR products obtained from 2 isolates from 2 regions on VP2, only LT-IPNV DNA produced with primers 2 and 3 could be sequenced by the former method. All remaining products were sequenced after cloning.

Sequence analysis of the LT-IPNV PCR product from nt 476-674 (Figs. 4.7 and 4.8) and its comparison with that of Ja IPNV revealed that there was a homology of 81.6% in nucleotide sequence but 100% in amino acid sequence. However, with AC IPNV a sequence of only 109 bp could be obtained,and this was from a region which could be aligned only with the primers (Fig.4.9). Similarly, with the LT-IPNV sequence obtained from nt 1220-1502, there was 80% homology in nucleotide sequence but 100% in amino acid sequence from nt 1200-1300 as

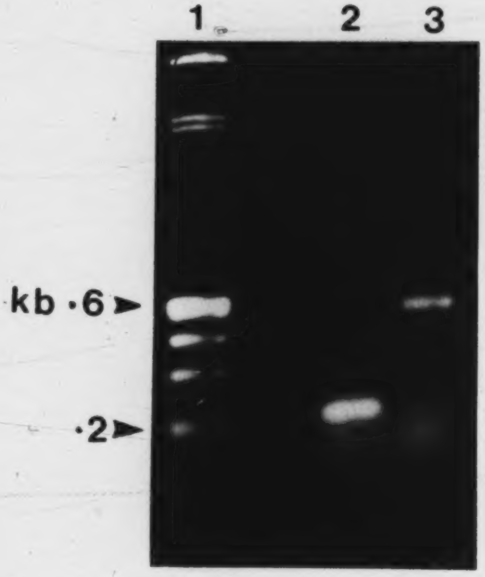

Fig. 4.4. Amplification of DNA after separating LT-IPNV PCR products in low melting agarose gel. Approximately one third of the DNA from I PCR was electrophoresed in 1.5% low melting agarose, and ethidium bromide stained bands were isolated under uv light. The isolated products were further amplified by PCR and electrophoresed in 1.25% agarose gel. The gel was stained with ethidium bromide and photographed by UV illumination with polaroid type 667 film. Lane 1, DNA markers; 2, 200 bp DNA ; 3, 600 bp DNA.

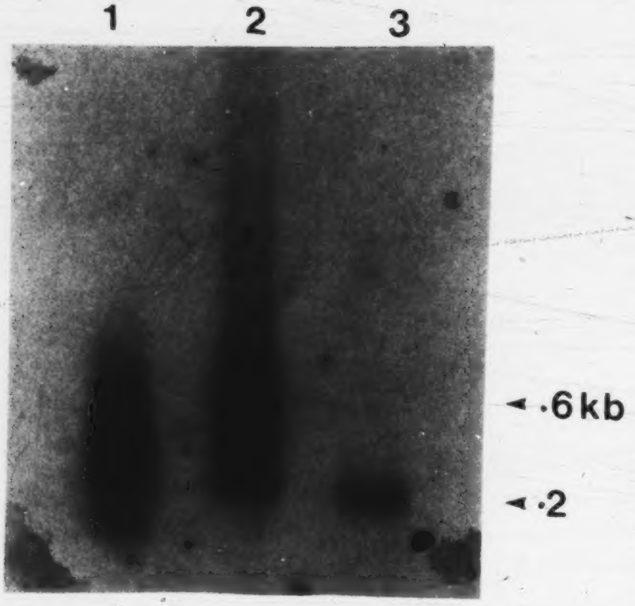

Fig. 4.3. Radioautograph of ^{32}P-labelled PCR primers hybridized to a Southern transfer of PCR products obtained using primers 2 and 3. Approximately one third of the PCR-amplified DNA was electrophoresed in a 1.25% agarose gel and transferred to a nylon membrane. DNA in the membrane was hybridized with ^{32}P-labelled primers at 55°C and autoradiographed. Lane 1, Ja IPNV ; 2, LT-IPNV ; 3,AC-IPNV.

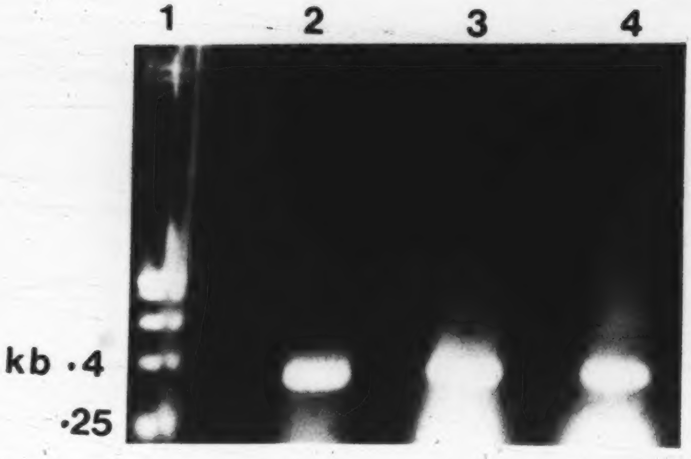

Fig. 4.5. PCR amplification of DNA from nt 1220-1502 of IPNV RNA using primers 4 and 5. cDNA synthesized using primers 4 and 5 by reverse transcription was amplified by 25 cycles of PCR. Each cycle in PCR consisted of a denaturation step at 94°C for 30 sec, an annealing step at 45°C for 1 min and and extension step at 72°C for 2-3 min. DNA after extraction with chloroform was precipitated in ethanol and electrophoresed in 1.25% agarose gel. The gel was stained with ethidium bromide and photographed. Lane 1, DNA markers; 2, Ja-IPNV; 3 LT-IPNV; 4, AC-IPNV.

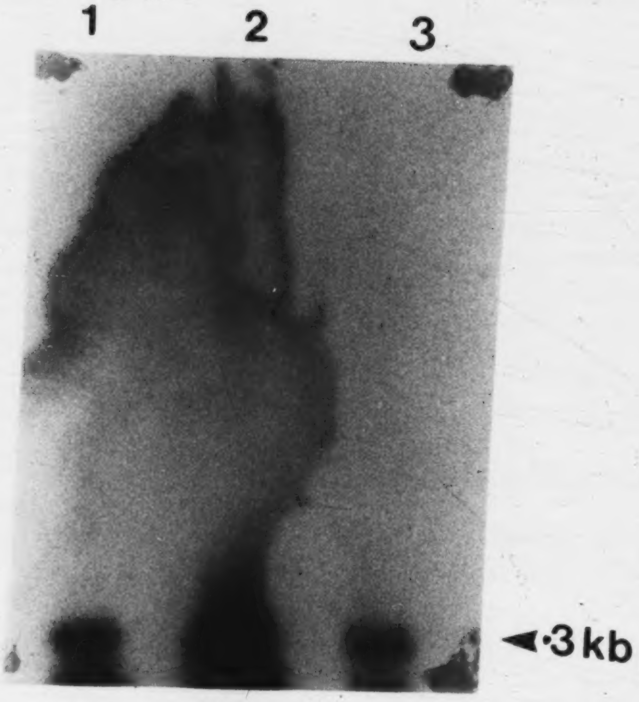

Fig. 4.6. Autoradiograph of ^{32}P labelled PCR primers hybridized to a Southern transfer of PCR products from primers 4 and 5. Approximately one third of the DNA from PCR was electrophoresed in 1.25% agarose gel and transferred to a nylon membrane. The filter was baked at 80°C for 2 h and DNA hybridized with ^{32}P labelled primers at 55°C, and autoradiographed. Lane 1, Ja-IPNV; 2, LT-IPNV; 3, AC-IPNV.

```
                                          nt476
                                           ▼
LT- GGCGNT---------------------------------ACATTCGAAGG -17
    :  : :                                 :: ::::::::
Ja- GCTGGTCTGTATGCACTCAACCGGACCCTGAACGCTGCCACCTTCGAAGG -50
LT- CAGTCTATCTGAAGTGGAGAGCCTGACATACAACAGCCTGATGTCACTCA -67
     ::::: :::::::: :::::::: :: ::::::::: ::::::: :: :
Ja- AAGTCTGTCTGAAGTAGAGAGCCTAACCTACAACAGCTTGATGTCCCTAA -100
LT- CAACGAACCCACAGGACAAGGTCAACAACCAACTGGTGACCAAAGGGATC -117
    :::: ::::::::::::::::::::::: ::::: ::::::::::: ::
Ja- CAACAAACCCACAGGACAAGGTCAACAATCAACTAGTGACCAAAGGAATT -150
LT- ACCGTCCTGAACCTACCAACAGGGTTCGACAAACCATACGTCCGACTAGA -167
    ::::::::::: :::::::: ::::: ::::: ::::::::::: :::::
Ja- ACCGTCCTGAATCTACCAACTGGGTTTGACAAGCCATACGTCCGCCTAGA -200
LT- GGACGAGACACCCAGGTCTC------------AGTCATGACGATAG -201
    ::::::::::::   :   :            :   :  : :   :
Ja- GGACGAGACACCACAGGGCCCCCAGTCCATGAACGGAGCAAGGATG -246
               ▲
             nt674
```

Fig. 4.7. Alignment of nucleotide sequences from nt 476-674 of Ja and LT-IPNV. The sequence for the A segment of Ja-IPNV is from Duncan and Dobos (1986). : indicates that residues are identical.

nt-476
Ja Thr Phe Glu Gly Ser Leu Ser Glu Val Glu Ser Leu Thr Tyr Asn Ser Leu
LT Thr Phe Glu Gly Ser Leu Ser Glu Val Glu Ser Leu Thr Tyr Asn Ser Leu

Ja MET Ser Leu Thr Thr Asn Pro Gln Asp Lys Val Asn Asn Gln Leu Val Thr
LT MET Ser Leu Thr Thr Asn Pro Gln Asp Lys Val Asn Asn Gln Leu Val Thr

Ja Lys Gly Ile Thr Val Leu Asn Leu Pro Thr Gly Phe Asp Lys Pro Tyr Val
LT Lys Gly Ile Thr Val Leu Asn Leu Pro Thr Gly Phe Asp Lys Pro Tyr Val

Ja Arg Leu Glu Asp Glu Thr Pro Arg Ser Gln Ser
LT Arg Leu Glu Asp Glu Thr Pro Arg Ser Gln Ser
nt 674

Fig. 4.8. Alignment of amino acid sequence of Ja and LT IPNV from nt 476 to 674

```
Ja- GCTGGTCTGTATGCACTCAATGGGACCCTGAACGCTGCCACCTTCGAAGG -50
    ::::: :::::::::::::::::: :::  ::: :: : ::: ::
AC- GCTGGGCTGTATGCACTCAATGGGCCCCCTAACTCTCCGACCCTCCTCCC -50
Ja- AAGTCTGTCTGAAGTAGAGAGCCTAACCTACAACAGCTTGATGTCCCTAA -100
AC- C------------------------------------------------- -51
Ja- CAACAAACCCACAGGACAAGGTCAACAATCAACTAGTGACCAAAGGAATT -150
AC- -------------------------------------------------- -51
Ja- ACCGTCCTGAATCTACCAACTGGGTTTGACAAGCCATACGTCCGCCTAGA -200
AC- -------------------------------------------------- -51
Ja- GGACGAGACACCACAGGGCCCCCAGTCCATGAACGGAGCAAGGATG -246
                      :   ::::::::::::::::::::::::
AC- ------------------CTAACAGTCCATGAACGGAGCAAGGATG -79
```

Fig. 4.9. Alignment of nucleotide sequences from 476-674 of Ja and AC-IPNV. The sequence for the A segment of Ja-IPNV is from Duncan and Dobos (1986).

```
    nt1197
    ▼
Ja- CCCAGTCAATCCTGACCGTGGCTGGGGTTTCCAACTACGAACTGATCCCC -50
    ::::::::::::::::::::::::: :: :::::::: :: ::::::::
LT- CCCAGTCAATCCTGACCGTGGCTGGCGTATCCAACTATGAGCTGATCCCA -50

Ja- AACCCAGACCTCCTGAAGAACATGGTCACAAAGTATGGCAAATACGACCC -100
    ::::::::::: ::::::::::::::::: :::::::: :: :: :::::
LT- AACCCAGACCTACTGAAGAACATGGTCACCAAGTATGGAAAGTATGACCC -100

Ja- GGAGGTGAACTACTATG----GATGATCCTATCACACAGAGAGGAACTGG -146
     ::::    : ::::::    ::::::::: :: ::::::::::: ::::
LT- TGAGGGCCTCAACTATGCCAAGATGATCCTGTCCCACAGAGAGGAGCTGG -150

Ja- ACATAAGGACAGTCTGGAGGACT-AGGACTACAAGGAGAGGACGAG-GTN -194
    :::: :: :: :::::::::::: :::: ::::: :: ::::: :: :
LT- ACATTAGAACCGTCTGGAGGACTGAGGAATACAAAGAAAGGACAAGAGCA -200

Ja- TTCAC-GAGATCACACTCTCGAGCGACTCTAGCTCAAGGCATGGCTGAGA -243
    ::::  ::::::::    :     ::::    : ::::  :      :
LT- TTCAAAGAGATCAC----T-----GACTT---CACAAGTGACCTACCAAC -238

Ja- GCCAAAGGCATGGGGCTGGAGAGACATAGTCAGAGGAATCCGAAAGGTCG -293
      ::::::::::::: ::::: ::: : :::::::: ::: :::: :: :
LT- CTCAAAGGCATGGGGATGGAGGGACCTGGTCAGAGGCATCAGAAAAGTGG -288

Ja- CAGCACCCGTCCTCTCAACGCTCTTCCCAATGGCGGCTCC -333
    : :: ::::: :: ::::::::::::::::::::::::::
LT- CCGCCCCCGTGCTGTCAACGCTCTTCCCAATGGCGGCTCC -328
                                           ▲
                                           nt1526
```

Fig. 4.10. Alignment of nucleotide sequence from 1197 to 1526 of Ja and LT-IPNV. The sequence for the A segment of Ja-IPNV is from Ducan and Dobos (1986). : indicates that residues are identical.

```
        nt 1202
Ja      Gln Ser Ile Leu Thr Val Ala Gly Val Ser Asn Tyr Glu Leu Ile Pro Asn Pro
LT      Gln Ser Ile Leu Thr Val Ala Gly Val Ser Asn Tyr Glu Leu Ile Pro Asn Pro

Ja      Asp Leu Leu Lys Asn MET Val Thr Lys Tyr Gly Lys Tyr Asp Pro Glu
LT      Asp Leu Leu Lys Asn MET Val Thr Lys Tyr Gly Lys Tyr Asp Pro Glu
                                                                nt 1302
```

Fig. 4.11. Alignment of amino acids sequence of Ja and LT IPNV from nt 1202 to 1302

compared to the Ja isolate (Figs. 4.10 and 4.11). From the same region with AC IPNV, a sequence of only 79 bp could be obtained which could be aligned only with one of the primers (Fig.4.12). From this sequence information it appears that less than half of the PCR amplified DNA from the 2 regions of AC IPNV was inserted in the vector during cloning. Alternatively, an artifactually amplified region, having no homology to the intended segment may have been preferentially cloned in each case.

4.4 DISCUSSION AND CONCLUSION

Earlier studies conducted in our laboratory indicated that the IPNV associated with lake trout is similar to that found in Arctic char, but distinctly different from the Jasper isolate in its VP2 polypeptide pattern and cross neutralization with rabbit antisera. However, LT-IPNV could be clearly distinguished from AC-IPNV by two binding and three neutralizing epitopes, as revealed by epitope analysis using LT-IPNV monoclonal antibodies. In the present study, however, the three isolates could not be differentiated by the mw of the RNAs. Earlier, RNA was used to distinguish the major IPNV serotypes AB, Sp and VR 299 (Macdonald 1981). Macdonald *et al.* (1982) also used the RNA to differentiate AC IPNV from two other IPNV isolates from eastern Canada. More recently, Hsu *et al.* (1989) used the genomic RNAs as markers to classify 11 IPNV isolates in Taiwan.

Initially, two PCR primers were designed to cover a 800 bp region in VP2, but a PCR product could not be obtained due to two probable reasons. First, the RNA was denatured at 95°C for 5 min before making cDNA at 50°C for 1 h during which the RNA strands might have reannealed. IPNV ds RNA is quite stable by virtue of the high stability of RNA-RNA double helices. Secondly, there may be

```
Ja- CCCAGTCAATCCTGACCGTGGCTGGCGTATCCAACTATGAGCTGATCCCA -50
    :::::::::::::::::::::::  :   : :   ::   ::
AC- CCCAGTCAATCCTGACCGTGGCTTCCAACTGCTCTTAAACGC-------- -42
Ja- AACCCAGACCTACTGAAGAACATGGTCACCAAGTATGGAAAGTATGACCC -100
AC- -------------------------------------------------- -42
Ja- TGAGGGCCTCAACTATGCCAAGATGATCCTGTCCCACAGAGAGGAGCTGG -150
AC- -------------------------------------------------- -42
Ja- ACATTAGAACCGTCTGGAGGACTGAGGAATACAAAGAAAGGACAAGAGCA -200
                                     ::: ::    ::: :::
AC- ---------------------------------AAGTAAAACTAAGTGCA -59
Ja- TTCAAAGAGATCACTGACTTCACAAGTGACCTACCAACCTCAAAGGCATG -250
    : :      : :  :   : : :       ::     :      :::
AC- TGCTTTTCAACCGATCGTTGCCCGCACACGCTCTTCCCAATGGCGGCTCC -109
```

Fig. 4.12. Alignment of nucleotide sequence from 1197 to 1526 of Ja and AC-IPNV. The sequence for the segment A of Ja-IPNV is from Duncan and Dobos (1986).

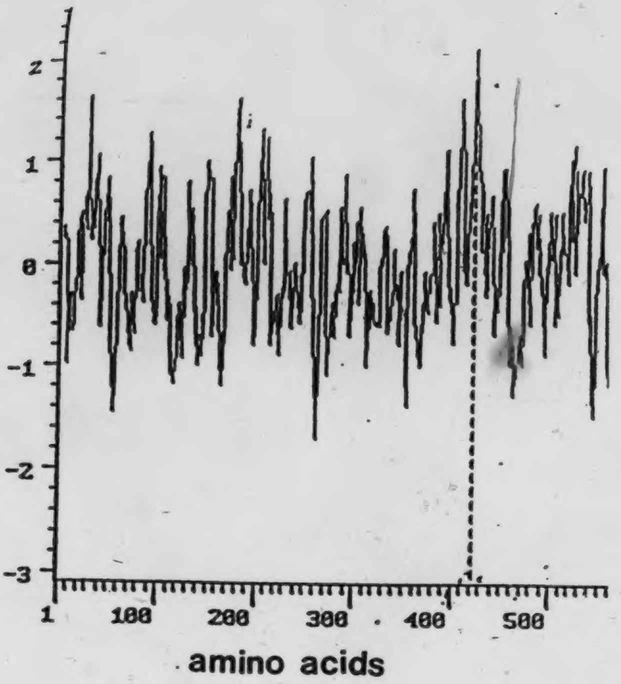

Fig. 4.13. Hydrophilicity profile of VP2 protein sequence of Ja-IPNV, computed using an average group length of 6 aminoacids. The amino acid sequence from 410 to 430 is indicated as the most probable antigenic site.

regions of secondary structures in the IPNV genome which hinder PCR. Later, when the interprimer spacing was reduced to 200bp and DMSO was used to help denature the RNA, a clear product was obtained. DMSO is known to prevent reannealing of RNA strands (Winship, 1989). PCR conducted with ds RNA of IBDV by Davis and Boyle (1990) indicates that 110 to 264 bp is an ideal length. However, Rimstad *et al.* (1990) could generate PCR product with IPNV with an interprimer spacing of 300 to 486 bp.

With primers 2 and 3, a 200 bp product was obtained with Ja, LT and AC-IPNV. But with LT-IPNV, in addition to the 200 bp product, a 600 bp product was also synthesized, although not consistently. However, in the case of Ja IPNV, the presence of an additional product of 400 bp was evident only after hybridization of a Southern transfer. These products could be artifacts occuring only with LT and Ja IPNV isolates because similar products were not seen with AC-IPNV, under the same PCR conditions. However, the bands from LT IPNV, when separated and amplified, generated the corresponding products, as seen in 1.25% agarose gel electrophoresis.

A distinct PCR product was synthesized with primers 4 and 5 only with Ja IPNV at an annealing temperature of 60^{o}C. A similar product could be synthesized with LT and AC IPNV only when the annealing temperature was reduced to 45^{o}C. This anomaly in PCR product formation at different annealing temperatures may be used for differentiation and specific identification of the isolates. It probably results from base mis-matches close to the 3' ends of the primers.

Sequence analysis of the LT IPNV genome from position 476 to 674 indicated a homology of 81.6% in nucleotide sequence and 100% in amino acid sequence with that of Ja IPNV. With the AC IPNV however, only 109 nt sequence could be obtained and aligned only to the primers region of Ja IPNV. Therefore another region in the VP2 gene with a likely difference between the isolates was sought. The region from nt 1200-1500 was chosen, because hydrophilicity mapping showed that the sequence from aa 410-430 (nt 1230-1290) is a highly antigenic site (Fig. 4.13) and hence isolates might differ in the region. When the sequence of LT-IPNV for the region 1202-1502 was compared with that of Ja IPNV a homology of 80% in nucleotide sequence was found. However, a 100% homology in amino acid sequence was found only from nt 1200 -1300. This region is highly antigenic according to the hydrophilicity map. However, with AC only 89 bases could be sequenced and this could be aligned only with the primers. This is due to a small insertion of PCR DNA in the vector which could not be explained. In the above cases a significant difference in nucleotide sequence, even though not resulting in a change in amino acid sequence, was seen between the isolates. Such a high mutation rate is characteristic of RNA viruses due to their shorter generation time and the error prone nature of RNA polymerases (Steinhauer and Holland, 1987). However, generally, substitution of nucleotides is four to five fold higher in the third position of codons compared to the first and the second, and this results in few changes in the amino acid sequence (Holland *et al.*, 1982).

In this study only two small regions (nt 476-674 and nt 1200-1300) of the total 1680 bp of the VP2 gene were sequenced. The squence data indicate that these regions are highly conserved in amino acid sequence between LT and Ja IPNV isolates and must have common epitopes. Haverstein *et al.* (1990) demonstrated that IPNV VP2 has two strongly conserved regions (the N and C termini) and a less conserved internal segment I. The I segment from aa 183-338 (nt 550-1014) is less conserved, and probably related to serotype specific epitopes. Heterogeneity was observed between Ja and N1 IPNV, and IBDV only in the internal segment I. In this study the internal segment was not sequenced and therefore differences between the isolates could not be seen. A complete sequence analysis of the VP2 gene of the LT and AC IPNV isolates will be useful to differentiate the two clearly at the gene level.

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CHAPTER 5

General discussion

Lake trout IPNV was first detected in 1984 in lake trout spawners from Cornwall Lake, which is in an isolated location in Northern Alberta, Canada (Larson, 1985). The virus was believed to be indigenous to the lake since salmonids have not been previously stocked in that region. Therefore, studies were made on the prevalence of the virus infection in adult lake trout, pathogenicity of the virus to other salmonids, and the antigenic and molecular relationship of the virus to other Canadian IPNV isolates.

Studies on the IPNV carrier rate showed that 44.4% of adult lake trout in Cornwall Lake are infected with the virus. This carrier rate was similar to the percentage of IPNV infection in Arctic char populations in the Mackenzie River Delta (Souter *et al.,* 1986). Virus in the spawners was detected most frequently in the pyloric caeca, less frequently in the intestine, and not at all in the kidney. In contrast, the virus in Arctic char was detected more frequently in kidney (49%) and caeca (43%) and to a lesser extent in spleen, liver and gonad (Yamamoto, 1989).

In experimentally infected brook trout fry, the virus caused typical symptoms of IPN and a mortality of 74% in 30 d. In these fish pyloric caeca, intestine, and gills were found to be the early sites of viral replication. The virus was less pathogenic to rainbow trout but it persisted in the infected fish for a period of 102 d when the experiment was terminated. The virus was not pathogenic to young lake trout , it's natural host. The virus did not cause mortality in two age groups of lake trout fry obtained from two different regions in Canada. However, as observed in rainbow trout, the virus also persisted in young infected lake trout. Hence the infection persists for a long time, possibly to the adult stage. It appears that lake trout IPN virus is non-pathogenic to its natural host but has low to high pathogenicity to other cultivable salmonid species. In contrast to LT-IPNV, the AC-IPNV caused 50% mortality in its natural host, the Arctic char and 35% in brook trout (Yamamoto, 1989).

LT-IPNV has 3 major proteins VP1 (96K), VP2 (51K) and VP3 (30K) contributing 3%, 61%, and 36%, respectively to the total virion protein as recorded with other IPNV isolates (Chang *et al.*, 1978; Macdonald and Dobos, 1981; Mertens and Dobos, 1983). The VP2 polypeptide characteristically showed two bands a and b contributing 44.32% and 16.67% respectively to 60.97% of the total virion protein. The two bands of LT-IPNV VP2 reacted with all of the MAbs in Western blot and hence it is likely that they have a common origin. Among the different IPNV tested in SDS-PAGE only VP2 of AC-IPNV showed two bands similar to that of LT-IPNV. However, LT-IPNV VP2 has a slightly lower mw (51K) compared to that in AC-IPNV (53K) and hence the two viruses can be differentiated in SDS-PAGE gels.

Cross-neutralization studies conducted using rabbit antisera indicated that LT-IPNV possesses minor antigenic differences with AC-IPNV but is distinctly different from Ja IPNV. Comparison of the mw of virion RNA to differentiate isolates as used in earlier studies (Macdonald and Gower, 1981; Macdonald *et al.*, 1983; Hsu *et al.*, 1989) indicated that there is no apparent difference in the mw of Ja, LT, and AC IPNV.

In order to resolve the antigenic differences between the closely related LT and AC-IPNV by epitope analysis, monoclonal antibodies were prepared to LT-IPNV. Fifteen MAbs were selected by virtue of their higher reciprocal additivity index (AI) values in ELISA (Friguet *et al.*, 1983), which indicated their specificity for different epitopes. All the fifteen MAbs reacted with the VP2 polypeptide in the Western blot but with different degrees, indicating the presence of both conformation and sequence dependent epitopes as observed in West Buxton and N1 IPNV serotypes (Caswell-Reno *et al.*, 1986; Christie *et al.*, 1990).

Epitope analysis by immunodot assay indicated that two MAbs, LT5 and LT13, reacted specifically with LT-IPNV,clearly distinguishing it from AC-IPNV. MAb LT4 reacted with both LT and AC IPNV, but not with C2, thus differentiating C3 from C2. Seven MAbs could recognize common epitopes on LT, AC and C2 which may indicate that the 3 isolates are antigenically closely related or share a common group antigen.

Among the 15 MAbs only 10 neutralized LT-IPNV. Three MAbs were specific only to LT-IPNV. One MAb which reacted positively by binding to LT, AC and C2 in the immunodot assay, could neutralize only LT-IPNV. Similar differences in binding and neutralization reactions have been noticed with MAbs to polio virus (Emini *et al.*, 1983) and IPNV (Caswell-Reno *et al.*, 1986). Seven neutralizing MAbs neutralized both LT and AC viruses, indicating the presence of large numbers of common epitopes on the virus isolates. LT3 and LT4 MAbs neutralized LT and C3 but not C2 viruses, which is similar to the immunodot reaction. Five MAbs neutralized LT, AC and C2 virus isolates. None of the LT-IPNV neutralizing MAbs neutralized Ja, BC1, C1 and VR 299 isolates. This pattern of cross reaction of LT-IPNV MAbs in neutralization in general was similar to that noticed in the immunodot test. Overall, using the panel of 15 MAbs, LT-IPNV could be clearly distinguished from AC, Ja and other Canadian isolates.

Recently, several IPN outbreaks have been recorded in trout hatcheries in Alberta. An immunodot assay performed using LT-IPNV specific MAbs LT5 and LT13 confirmed that the IPNV isolates have originated from lake trout from

Cornwall Lake. Propagation of fingerlings from lake trout eggs collected from Cornwall Lake and their distribution to other hatcheries and lakes in Alberta may have contributed to the spread of the virus.

In order to understand this relationship at the molecular level two regions of VP2 gene of LT and AC IPNV were sequenced and compared with that of Ja. The two regions from the genome were amplified by the polymerase chain reaction using specific primers selected from the VP2 sequence of Ja-IPNV. Anamaly in DNA synthesis between the isolates at different primer annealing temperatures was observed in PCR from nt 1202-1500. A distinct PCR product was synthesized only with Ja IPNV at an annealing temperature of 60°C. A similar product could be synthesized with LT and AC IPNV only when the annealing temperature was reduced to 45°C.

Analysis of the LT IPNV genome from nt 476 to 674 indicated a homology of 81.6% in nucleotide sequence and 100% in amino acid sequence with that of Ja IPNV. Further analysis and comparison of the LT-IPNV genome from nt 1202-1502 with Ja indicated a homology of 80% in nucleotide sequence. A 100% homology in amino acid sequence was found from nt 1200-1300. However, with AC IPNV sequences of only 109 and 89 bases could be obtained from the 2 regions and hence could not be compared with either Ja or LT IPNV.

Sequence homology in the above two regions agrees with the findings of Haverstein *et al.* (1990) who demonstrated that IPNV VP2 has two strongly conserved regions (the N and C termini) and a less conserved internal segment I from aa 183-338 (nt 550-1014). In this study the internal segment was not sequenced and therefore differences between the isolates could not be seen. A complete sequence analysis of the VP2 gene of the LT and AC IPNV isolates will be useful to clearly differentiate the two at the genomic level.

From the above studies it can be concluded that LT-IPNV persists in 44.4% of adult lake trout in Cornwall Lake. The virus persists mainly in pyloric caeca and is non-pathogenic to its natural host but pathogenic to other cultivable salmonids. The virus differs from AC-IPNV with respect to the site of persistence of virus and pathogenicity to its natural host-the lake trout. The virus has a characteristic VP2 polypeptide consisting of 2 bands similar only to that of AC-IPNV. However, the VP2 of LT-IPNV differed in having a lower mw (51K) compared to 53K in AC-IPNV. The virus could be further differentiated from AC-IPNV by two binding and three neutralizing monoclonal antibodies. Overall, the virus could be distinguished from other Canadian IPNV isolates using the panel of 15 LT-IPNV MAbs. Therefore, the virus is antigenically distinct from AC and Ja and other Canadian IPNV isolates and is indigenous to Cornwall Lake. However, the virus could not be differentiated from Ja IPNV in the amino acid sequence of the two regions in VP2 that were sequenced.

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APPENDIX

10X TEB: 1 M Tris, 1 M Boric acid and 20 mM EDTA.

TE buffer: 5 ml of 1 M Tris-HCl pH 8 and 1 ml of 0.5 M
in 494 ml H_2O.

2X YT:
16 g/l bacto-tryptone
10 g/l yeast extract
5 g/l NaCl
about 15 g/l bacto-agar for plates, 6g/l for sloppy agar
Add H_2O to 1 l and autoclave. Cool the medium to about
50^{o}C and then add ampicillin at 100 ug/ml.

Lysozyme solution:

glucose	50 mM
EDTA	10 mM
Tris-HCl	25 mM(pH 8)

Add dH_2O to 100 ml and store at 4^{o}C.

Lysis solution:

NaOH	0.2 N
SDS	1%

Add dH_2O to 100 ml and store at RT.

Potassium Acetate :
(pH 4.8)

5 M KOAc	60 ml or 29.45 g to make up 60 ml solution
Conc. HOAc	11.5 ml
dH_2O	28.5 ml

store at 4^{o}C.

20X SSPE:
3 M NaCl
0.2 M NaH_2PO_4-H_2O
0.02 M Na_2 EDTA
Adjust pH to 7.4 with NaOH.

20X SSC : 88.23 g Na-citrate/l and pH 7.

6% polyacryl amide gel :

38:2 acrylamide solution	11.25 ml
10X TEB	4.5 ml
Urea	37.5 g
dH_2O	to 75 ml

2 ml for plug, add 5 ul 20% AP and 4 ul TEMED. for the rest, add 250 ul 20% AP and 10-15 ul TEMED

Dilute 10X TEB (1M) to 60 mM for running buffer.

Sequencing loading buffer: (stop mix dye)

80% (v/v) deionized formamide
10 mM NaOH
1 mM EDTA
0.1% (w/v) Xylene cynol
0.1% (w/v)bromphenol blue

10 X PCR buffer: 700 mM Tris-HCl,pH 8.8, 20 mM $MgCl_2$, 1% Triton X-100.

Tris buffer saline: (TBS) 50 mM Tris-HCl, 200 mM NaCl, pH 7.4.